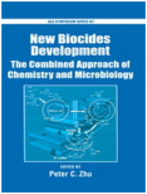

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New Biocides Development

The Combined Approach of Chemistry and Microbiology

EDITED BY

Peter C. Zhu

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Peter C. Zhu, Editor
ALZA, Johnson and Johnson

American Chemical Society, Washington, DC

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

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Preface

Biocides, in the form of disinfectants, preservatives, and, if appropriately used, sterilants, have been at the forefront of healthcare for more than a century. Such molecules and their application to hygiene gave enormous reductions in sepsis, and together with a growing knowledge of the interplay between microorganisms and disease contributed greatly to our health expectancy. Antibiotics are relatively new kids on the block, but after 50 years of use they are waning in their effectiveness. New antibiotic strategies and molecular classes are required as resistance development issues come to the fore. Sadly, development of new antibiotics does not present the same profit line as therapeutic avenues for chronic clinical conditions. There is little evidence that the effectiveness of biocides has reduced with time, but increased awareness of general toxicological issues and concern for the environment has dictated against some chemical groups of reactive biocides. There is a need for refinement and new fundamental research to develop cheap, effective, and safe molecules for application outside of therapeutics. Historically, it was the interplay of chemistry and microbiology that helped to create new biocides and new methods of infection control. Recent progress in both chemistry and life sciences once again offers many opportunities and challenges in these areas. This book reflects a symposium held in Honolulu, Hawaii in December 2005 that brought together many scientists from industry and academia representing engineers, chemists, microbiologists, and physiologists to review recent cross-disciplinary developments in biocides and infection control. The focus of the meeting was on (1) newer and safer biocidal chemicals for infection control, (2) mechanism(s) of the biocidal action of chemicals against planktonic forms and biofilms of pathogenic organisms, and (3) biocides directed against emerging, reemerging, and

In New Biocides Development; Zhu, P.;
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antibiotic-resistant pathogens. This was the first symposium of its kind at a PacifiChem conference, but judging by its success, will not be the last.

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Chapter 1

Effects of Environmental Chemicals and the Host–Pathogen Relationship: Are There Any Negative Consequences for Human Health?

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The potential impact of environmental chemicals on humans and the environment is well recognized by the public and is supported by scientific data on a variety of species, including humans. However, the impacts of chemical exposures on the initiation and progress of infections, and on the microorganisms that cause them, are much less acknowledged. We review the data that support a role for environmental chemicals in augmenting the infectious process through effects on host, as well as directly through chemical exposure of microbial pathogens The cumulative data clearly indicate that this topic warrants further investigation.

Introduction

> "There are in nature neither rewards nor punishments – there are consequences."
>
> ***Robert G. Ingersoll***

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Living cells, whether microbial or human, are acutely tuned homeostatic systems that inevitably interact with their environments on a continuing basis. The extent and nature of these interactions obviously varies depending on the species and niche(s) that are occupied, but the results of such interactions can be positive, negative or neutral. As a species, humans live in a variety of environments, but increasingly the largest human populations are found in cities that are centres of employment and hubs of industrial activity. Microorganisms as a group are even more ubiquitous. Though particular environments contain microbial communities with potentially large numbers of species; some types/groups may predominate or become dominant under external influences. Their small size and behavior make microbes both highly sensitive and very responsive to their external surroundings.

Since the beginning of the industrial revolution, both humans and microorganisms have come into close contact with increased levels and types of chemicals, many of which are not found in significant concentrations, if at all, in nature. Early occupational exposures to chemicals in particular industries were documented to result in diseases such as silicosis of miners and industrial workers exposed to silica dust; testicular cancer in chimney sweeps exposed to soot; neurological disease in potters exposed to lead glazes and in hat makers exposed to mercury; and bone disease in workers exposed to phosphorus in the manufacture of matches. For many, such continuous or intermittent exposure took place against a backdrop of sub-standard living conditions and poor hygiene.

Fast forward a century or so, and in industrialized countries workplace exposures have dramatically dropped with occupational health and safety as a priority issue. Significant improvements in living standards and hygiene have also occurred, though in some cases routine institutional and domestic practices have jeopardized these improvements (*1*). Although direct occupational exposures to chemicals have been largely controlled, there is a greatly increased environmental use of chemicals. Along with a large number of technological advances, the public was promised "better living through chemistry", and indeed lives have for the most part improved. In 1962, Rachel Carson's ***Silent Spring*** (*2*) exposed the hazards of the pesticides such as DDT, and more recently the awareness of hormone mimics and disruptors (*3*) has come to public attention. While both of these events have transformed public awareness, they in fact represent only a small fraction of human-chemical encounters. Human exposure to environmental chemicals is now ubiquitous and insidious in air, water, food and many consumer products, and the major routes of exposure are therefore ingestion, inhalation and through exposed surfaces. Similar exposures can be expected for domestic animals and crops as well as wildlife.

Most evidence of the health and environmental effects of xenobiotics and even naturally-occurring chemicals on biological systems has come from tests carried out one chemical at a time. While this approach makes regulatory tools simpler, it does not represent real environmental exposures to mixtures of chemicals. This potential complexity has been recognized, but is rarely discussed or examined (*4-6*). Systemic or genotoxicity of chemicals is assessed through bioassays (*7*) ecosystem effects (*8*) and or quantitative risk assessments (*9*), though given the large numbers of environmental chemicals, only a fraction of these have been tested adequately. To an increasing extent, therefore, toxicity is predicted based on chemical formulation and relationship to other tested chemicals (*10-12*). Chemical genotoxicity (mutagenicity) is often examined on microbial systems (*13-15*) but the logical extension of such tests to study the evolution and potentially altered virulence of pathogenic microbes is not addressed.

Moreover, if a susceptible host is involved in simultaneous or sequential exposures to both infectious agent(s) and chemicals, the effects of one or the other, or both, may be enhanced. In spite of the clear potential for such augmentation or synergisms between chemicals and infectious agents, the impetus for examining such combined exposures has been largely lacking, and relatively little literature exists for measuring such combined effects at realistic exposure levels in animal models.

Figure 1 shows a compartmentalized model of potential interactions among humans, microorganisms and chemicals. It should be viewed against a backdrop of human infections caused by a variety of microorganisms. The closed boxes show results that may be expected whereas the statements crossing or below the arrows show the influences from the compartment where the arrow arose. The dynamic and statistical nature of such interactions also needs to be emphasized. This review will discuss aspects of these interactions as illustrated in the human and microbial compartments, drawing on published data where it is relevant, and at times speculating on some likely but undocumented occurrences. The effects of microbial interaction with chemicals on the environment will not be discussed.

It is beyond the scope of this review to address in any detail the toxic effects of the chemicals themselves which are amply documented. Nor is it within our purview to examine interactions among microorganisms and their hosts as to whether one infection could predispose to a second, or exacerbate symptoms of a disease. Specifically, we will address two aspects of the problem: a) the chemical modulation of host functions and immunity that may, in turn, alter susceptibility to infectious agents (indirect effects), and b) the direct effects of chemicals on microorganisms in the environment that may change their survival, pathogenicity, tropism or response to environmental or therapeutic control measures. As noted above, it is likely that many plant and animal hosts may encounter similar issues, but our focus in this review is on human health effects where these are known. Nevertheless, examples of studies from other species are included in the absence of evidence for humans and where these are germane to the discussion.

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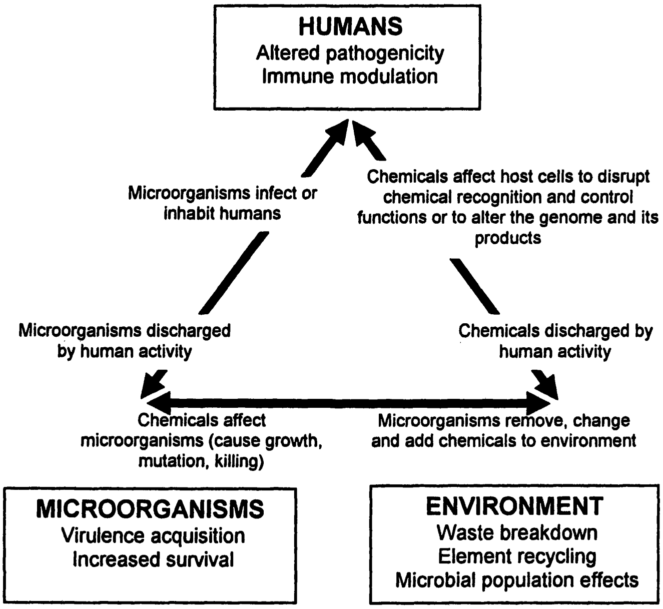
HUMANS
Altered pathogenicity
Immune modulation
Microorganisms infect or inhabit humans
Chemicals affect host cells to disrupt chemical recognition and control functions or to alter the genome and its products
Microorganisms discharged by human activity
Chemicals discharged by human activity
Chemicals affect microorganisms (cause growth, mutation, killing)
Microorganisms remove, change and add chemicals to environment
MICROORGANISMS
Virulence acquisition
Increased survival
ENVIRONMENT
Waste breakdown
Element recycling
Microbial population effects

Figure 1: Potential interactions between environmental chemicals, microorganisms and humans

Setting the Stage

Chemicals, their sources and routes of exposure for humans

Environmental chemicals come from a variety of sources. Traditionally, these are perceived by the public to be from industrial (heavy metals, polyaromatic hydrocarbons – PAH, polychlorinated biphenyls – PCB, etc.), or agricultural (pesticides, herbicides, nitrate, etc.) sources. However, increasingly there is a realization that the sources of toxic chemicals are much broader and encompass not only anthropogenic chemicals from heavy industries but also from consumer items and personal-care products, drugs of many types, metabolites from breakdown of complex organics and even natural products produced as toxins by bacteria, fungi, algae and plants. Such natural toxins may be exported from cells (exotoxins) or held within the cell as a part of the cell structure (endotoxins). The latter are exemplified by bacterial endotoxins that comprise part of the cell walls of Gram-negative bacteria and which are ubiquitous and significant pyrogens that need to be excluded from any products for parenteral administration. Although a wide range of chemicals can be

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considered as environmental and or health threats and, generally, good analytical tools are available, the exact nature of most environmental threats and mixtures of chemicals is ill-defined.

The health and environmental impacts of chemicals can be acute or chronic depending on the levels and frequencies of exposure. The chemical structure, lipophilic nature, binding properties, and potential for degradation or clearance of the chemical tend to determine whether it accumulates in exposed cells and tissues. Risks from chemical exposure usually increase with dose, though not necessarily linearly, and sometimes even low doses trigger a significant host response.

Since the impact of environmental chemicals was recognized, the amounts of a number of persistent chemicals released in industrial wastes have been abated significantly, though they remain as global contaminants. Even in agriculture there is a trend away from persistent pesticides to those that may be more toxic initially, but which are more readily degradable. The tendency for overall chemical exposures to increase is however unabated with the main drivers of population increase, urbanization, industrial production and consumerism, increased waste production and disposal, and, last but not least, the fear of infection.

This last factor is an important one; it has driven consumption of antibiotics over the last few decades and that trend is now being actively opposed to try and reduce the global prevalence of antibiotic-resistant bacteria (*16, 17*). However, global production, marketing and dispersal of biocidal chemicals is still increasing as their use goes far beyond the needs for microbial control in aspects of medicine, agriculture and food production, and into the preservation of infrastructural materials and consumer products; it has become difficult to purchase personal-care products that do not contain antimicrobials. The global market for biocides has grown 40% since 1992 and is still increasing about 4%/yr (*18*).

While chemicals from industrial, agricultural, institutional and domestic waste sources usually reach humans at relatively low concentrations (often ppb or low ppm), those in products used directly by the consumer are usually at rather high levels (high ppm to low ppt). Consumer exposure is predominantly through a plethora of drugs (including antibiotics), cleaners, disinfectants, antiseptics and garden pesticides. In turn, as such drugs fail to be absorbed by patients, or chemicals are discharged from the domestic environment, they also end up diluted in what we eat, drink and breathe. Analysis of municipal wastes can clearly show the presence of a number of such compounds including antibiotics (*19*), steroids (*20,21*) other drugs (*22-24*) antimicrobials, preservatives, and personal-care products (*21,24*). Levels of trihalomethanes (THM) in drinking water are strictly controlled because of potential carcinogenicity (*25, 26*), yet the levels of chloroform in breath and blood can be monitored readily during and after performance of household water-contact functions (*27*). An interesting observation in pulp mill wastes, which are often

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mixed with sewage, is that progesterone in the waste can be converted to androgens by bacteria in the wastewater (*28*). The application of sewage sludge and other organic wastes to agricultural lands is a hotly debated issue, but few realistic alternatives exist for safer disposal.

Food, water and air are the principle and obvious vehicles for delivering chemicals, and indeed microorganisms, to humans. Food, whether animal or vegetable, can accumulate chemicals and potentially harmful microorganisms prior to its consumption. Distribution of chemicals in the fluid vehicles (air and water) is not uniform. Like microorganisms, many chemicals tend to attach to surfaces. In fact, one of the explanations put forward as to the propensity of microorganisms on surfaces is that it is advantageous for them to be there when nutrient sources are low because nutrients accumulate on surfaces. Thus particulates suspended in air and water can theoretically deliver to the host, simultaneously or separately, both chemicals and pathogens in microscopic 'packages'.

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Microorganisms, host-microbe interactions and vulnerable host systems: the indirect effect of chemicals on infections

The diversity of microorganisms in the environment and even in human hosts is enormous. For example the human gut has been estimated to contain of the order of 500-1000 commensal microbial species (*29*) and ten times as many cells as comprise the human body. Each individual has their own complement of microbial species established early in life. Transient exposure to pathogenic microorganisms can produce dramatic health effects. Infections can be either acute or chronic and localized or systemic, with microorganisms replicating in different target tissues to varying degrees or not at all. Microorganisms are complex and much more difficult to work with than chemicals. Also, because of their size, they would generally be excluded by intact and functional epithelial barriers (skin and mucous membranes). Infections initiated at mucosae that compromise cell integrity can allow the invading microbe access to the circulatory system and underlying tissues.

Humans, like many other organisms, have highly complex immune systems with a variety of cells integrating a concerted and co-operative defence against invading pathogens. Many components of this system are present at the epithelial barriers, especially mucosae, and are hence exposed and vulnerable to environmental influences, including both chemicals and microorganisms. In contrast to chemical exposures, for a healthy individual with intact adaptive and innate immune responses, the risk for infection with the same organism generally declines with exposure. However, a compromised immune system can lead to increased susceptibility to infection as well as inappropriate responses to specific antigens (autoimmunity, hypersensitivity).

The many cell types in the immune system and their integrated communication and functionality are perhaps what make it so vulnerable to damage, rather like a very complex computer program. Inter- and intracellular signaling is mediated by secreted chemicals that interact with specific receptor molecules. Binding of xenobiotics, or even other natural products and toxins, to signal molecules or receptors can theoretically disrupt many aspects of immune function. Chemicals can also compromise membranes, interfere with enzyme activities and potentially cause a number of different and simultaneous effects based on the statistics of cell exposure. Effects could also occur at a range of scales depending on the target(s) affected - from a very local level for a single infection with no sequelae to a more generalized and potentially damaging effect if immune responses are compromised. Genotoxic chemicals could also affect the formation, differentiation, distribution and longevity of immune system cells on a permanent basis.

Thus it may be easy to understand how, in general terms, exposure to chemicals could make individuals more susceptible to infection. At the same time, the reverse may also apply; tissue damage from infections could facilitate chemical access local to the infection and even into the circulatory system. How significant such potential synergisms are in reality is difficult to discern and has not been widely explored in animal models.

The response of the immune system to infection is clearly affected by host age, and genetics. Very young children with immature immune systems are vulnerable to infections. As the immune system matures the ability to cope with infectious agents increases but so does the exposure during childhood as 'new' pathogens are encountered. Chemical exposures during these vulnerable years could have life-long impacts on health mediated through immune function as discussed above. Aging adults also become more sensitive to infections; though the exact reasons for this are less well understood, it is sometimes suggested that accumulated replication errors or results of 'encounters' with mutagens could be at least partly responsible for the aging process. Although the role that exposure to environmental chemicals may play in this process can only be guessed at, it clearly could be a contributing factor.

Even if it is the most complex and pertinent to infectious agents, the immune response is not the only host function that could be compromised by combined or sequential exposure to chemicals and microorganisms. For example, the endocrine system is exquisitely sensitive to endogenous hormones and increasing numbers of environmental chemicals have been shown to potentially be able to mimic or disrupt this (*30*). Nevertheless, the link with infection has not been demonstrated though during childhood and developmental years it remains as a possibility.

Chemicals that can cross the blood-brain barrier could potentially interact with infectious agents to affect neurotransmission and brain as well as motor functions; Parkinson's disease and other neurodegenerative disorders could be examples (*31, 32*), but the evidence is inconclusive. Nevertheless, chemicals

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could cause reactivation of latent virus infections, and so the question of whether there could be a role for these combined effects in chronic and degenerative disorders is pertinent.

Another example of where interactions of chemicals and infectious agents could be highly important, though largely unproven in combination, is the developing fetus. There is considerable evidence for chemical effects in utero (*33*) and also for viral or bacterial infections that can result in termination (*34*). One could only speculate as to whether combinations of chemical and biological assaults could combine to cause fetal abortion or abnormalities. Here also it is the extreme complexity and sensitivity of the development and differentiation that must take place *in utero* that makes the system potentially vulnerable.

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What is the evidence for combined effects of chemicals and pathogens on the host?

What follows is a commentary on the state of knowledge in this area. The literature behind health effects from chemicals is vast and overwhelming. One could perhaps assume that any modulation of immune system functions could automatically be expected to have implications for infection risks but that would be unreasonable and overstating the argument. Extracting such evidence from the literature has been a little like unraveling a tangled ball of string. There is a lot of fragmented information but only a little can be gleaned at a time. Search terms lead to chemicals or microorganisms but rarely both. For example, the role of Toll-like receptors (TLR) in pathogen recognition is now well recognized and so modifications of TLR may be important, however, a search of electronic journals for TLR and environmental chemicals brought no hits. There are a number of model experimental systems used to study infections that deliberately use chemicals as irritants or to compromise cell and tissue functions, but these are totally contrived and unlikely to occur naturally.

If one asks the question of how simultaneous or sequential exposure to chemicals and infectious microorganisms may result in synergistic or additive effects on the exposed individual, then there are a lot of options in the answers, but it becomes obvious that the risks of such combined effects must increase with frequency of exposure to either or both types of agent. This in turn suggests that chronic chemical exposures and relatively common infections are the most probable culprits. We have therefore selected a few key areas where knowledge can be summarized more meaningfully. These include airborne pollutants and particulates and respiratory infections, food- and waterborne contaminants and gastrointestinal pathogens, heavy metals since exposure to them is often localized and chronic, infections associated with drug administration, and viruses and chemicals as co-carcinogens. This selection is not the total sum of evidence for the phenomenon but is simply what can most easily be presented.

Air pollution

Since humans have their greatest environmental contact with the air that must be involuntarily breathed for survival, it is perhaps not surprising that most of the evidence for combined human health effects is related to airborne pathogens and particulates. Lung structure and function is designed to clear many of the particulates that it encounters during breathing and clearance mechanisms must remain fully functional for optimal discharge of pollutants of all types. Practices such as cigarette smoking may impair particulate clearance. Such clearance is backed up by the innate and adaptive immune mechanisms in the lung tissues, and antimicrobial peptides in nasal secretions. Nevertheless, especially in cities degraded air quality in the streets and buildings bombards the lungs with volatile chemicals and particulates that can impair respiratory function. Furthermore, in the same crowded environments humans are readily exposed to infectious agents shed by other humans in the breathed air as well as after infectious particles have settled onto surfaces.

Gases emitted from burning of fossil fuels in automobiles and industries, formed during exposure of certain chemicals to sunlight, or volatilized organics from a variety of sources are among the most important and best recognized airborne pollutants. Of these, sulphur dioxide, nitrogen dioxide and ozone are perhaps the most prominent and well known in our cities, but there are many unknown or unrecognized chemicals also. The automobile alone can contribute the vast majority of airborne particulates in some cities. From the standpoint of human inhalation, the most significant particles that are not cleared from the lungs are those in the respirable range (<2.5 μm).

One may anticipate that particulates laden with chemicals and microorganisms may have cellular and thereby immune system effects, perhaps predisposing the exposed individual to new infections or exacerbating pre-existing ones. There is some evidence that children exposed to airborne pollution are more vulnerable to infection (*35*) and that environmental oxidants can influence the course of a respiratory virus infection as well the associated inflammation (*36*). There has been a particular interest in diesel exhaust fumes, partly because of the esthetics, but indeed diesel exhaust has been shown to decrease immunity to *Listeria monocytogenes* (*37*) and experimental animals exposed to diesel exhaust had decreased ability of the lungs to clear bacteria, decreased ability to produce interferon and increased viral multiplication in the lung (*38*); they state, “These results support the hypothesis that exposure to diesel exhaust particles increases the susceptibility of the lung to infection by depressing the antimicrobial potential of alveolar macrophages. This inhibitory effect appears to be due to adsorbed organic chemicals rather than the carbonaceous core of the diesel particles.” Other studies in animals have shown that many particulates including cigarette smoke, acid aerosols, metals, organic compounds, and combustion products can interfere with the normal defense

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processes of the lung and thereby enhance susceptibility to respiratory infection or exacerbate allergic diseases (*39*).

Such detrimental effects of exposure to pollution are not easy to quantify in humans, but the perception and probable reality is that exposure to poor air quality and pollutants in cities makes us more vulnerable to infection. The dramatic rises in the prevalence of asthma and atopy in recent years may or not be strongly associated with exposure to airborne contaminants. However, asthma exacerbations are commonly associated with cold symptoms with or without additional exposure to chemical pollutants (*40*).

Chronic obstructive pulmonary disease (COPD) has also shown a steady increase for which attribution has not been determined, but a role for airborne contaminants is not unlikely and chronic airway infection is likely to co-exist because of impaired clearance functions (*41*). Hypersensitivity pneumonitis has been associated with a number of different triggers including exposure to certain microorganisms. All such conditions could readily be affected by combinations of chemicals and microorganisms in a complex manner, possibly depending on whether the exposures are simultaneous or sequential, and in which order.

Genotoxicity of outdoor air has been reviewed (*42*) as well as the specific genotoxic effects of sulphur dioxide on mice (*43*). While these do not relate to infection directly, the potential for mutations exists in either the infectious agent itself or the host. It is generally recognised that indoor air quality (IAQ) reflects that of local outdoor air though because of reduced circulation, the quality tends to be much lower. There is considerable interest in the effects of IAQ on humans who spend a large part of their day working in environments with recirculated air. Indoor environments are also likely to contain higher levels of endotoxins, infectious agents, tobacco smoke, wood smoke, and, in new buildings, volatile chemicals from building materials. Dampness in indoor environments has been associated with allergic symptoms that could be triggered by multiple microbes and mold toxins (*44*).

There is also considerable evidence for effects beyond humans. Increases of parasitism (up to 80%) of soil invertebrates by protozoa have been correlated with airborne pollutant levels whereas much lower rates (0-20%) were observed at uncontaminated sites (*45*). Heagle (*46*) and Manning (*47*) have comprehensively reviewed the effects of pollutants on plant infections. One can therefore conclude, perhaps, that airborne chemical pollutants can have significant impacts on the infectious burden of many species that live in the immediate environment or downwind from pollution sources. That our cities and their inhabitants may bear the brunt of these impacts is also not an unreasonable expectation.

A recent study relevant to airborne pollution has demonstrated that ultra-fine particles in the lungs can cross the cell membrane by non-phagocytic means and acquire intracellular access through diffusion and adhesion to cellular constituents (*48*). If one can envisage the microscopic particulate package of

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chemical and pathogen, then this may help to provide a partial explanation for some of the clinical observations of combined effects.

Water and food pollution

Essentially all chemicals that are present in the air can be deposited onto soils or into waters during precipitation that scrubs the air of its chemicals and particulates. It is thus not surprising that a relatively similar spectrum of the more persistent chemicals can be found in food and water. Furthermore, atmospheric photochemical transformations may increase or decrease the toxicity of the pollutant mixture. When this is coupled with global air movements, and documented accumulation of pollutants in areas remote from pollution sources, one can envisage far-reaching ramifications of chemical pollution on the ecosystem and also on humans.

Furthermore, in water and foods there is a broader spectrum of potential bacterial and fungal pathogens, and their persistence and augmentation is possible and even likely under appropriate conditions. Foodborne infections are amply documented. As for the chemicals, there are recognized accumulations of certain toxicants in foods and waters and demonstrations of chemical accumulation and transformation in soils used for food crops. There are also demonstrable effects from toxins in foods, either intrinsic to the food, accumulated because of the environment of production, or acquired perhaps because of conditions of storage (e.g., aflatoxins). In spite of all this, the evidence for combined effects of chemicals and infectious agents is not well documented outside aquatic species and it was difficult to find examples relevant to humans to cite in this section.

As discussed above, there is a real potential in foods and waters for simultaneous exposures to microorganisms and chemicals as a package. Pesticides, fertilizers and sometimes antibiotics are widely applied to soils and food crops, and domestic animals are husbanded with antibiotics, hormones and drugs. When such foods are also contaminated by pathogenic microorganisms, potentially in large numbers, then combined exposures are almost certain.

In addition to water receiving chemicals scrubbed from the air, it also receives all the land run-off from farming operations as well as industrial, institutional and domestic wastes. Genotoxic contaminants in potable water include metals, low levels of pesticides, PCB's, PAH, disinfectant residuals, disinfection byproducts, algal toxins, etc. (*21*).

There is speculation that the mass decline observed among amphibians worldwide may be due to infections (*49*), perhaps facilitated by host immune system effects or enhanced by persistent chemical pollutants acting in synergy or directly on the microbial pathogens to increase their persistence and virulence. Similarly either chemicals or infection or both could be responsible for increases in amphibian deformities (*50*). Chronic infections in turtles have been shown to be associated with increased levels of organochlorine pesticides

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(*51*). One could suggest that amphibians, and potentially reptiles, may be particularly vulnerable because of both aquatic and terrestrial exposures, but further evidence is needed in this regard.

Khan and Thulin (*52*) have tried to pull together a body of evidence suggesting the parasites of fish can be used as pollution indicators because they see a greater severity of parasite impacts on fish in conjunction with the presence of a variety of pollutants including domestic sewage, pesticides, PCB's, heavy metals, pulp and paper effluents, petroleum aromatic hydrocarbons, etc. They cite a number of experimental studies supported by field data that imply immune system effects on the fish. This is supported by more recent evidence highlighting the inflammatory responses of fish infected with parasites and exposed to pollutants (*53*).

Beluga whales in the St. Lawrence River estuary are among the most contaminated mammals studied; at necropsy, these animals showed, in addition to the chemical contaminants, severe disseminated infections rather than just mild pathogens (*54*). The possibility of severe immune system effects from pollution, including organochlorine compounds, is considered. Inhibition of functional activation of macrophages can decrease resistance to infection by some of the bacteria most capable for causing disseminated infections such as mycobacteria (*55*).

Even plants are not immune to the combined effects of pathogens and pollutants, with infection being greater according to the degree of pollution (*56*). In the case of plants, the combined air, soil and water effects are probably confounded, though they make ideal study subjects in experimental exposures because their sedentary habit means they can be placed exactly and effects can be categorized in terms of pollution gradients.

It would be only speculation to suggest that any increase in human infections is directly related to combined chemical and microbial exposures in food and water above and beyond what may occur with the microorganisms alone. There is little evidence for this, especially if one considers that effects are often mediated through the immune system. However, if one considers that an individual cell could be exposed to a 'package' of pathogen and chemical; does this combined assault make an infection more likely? We leave this possibility to the reader's best judgment. We found only one study that examined the potential effects of organochlorine exposure during childhood and infections (*57*); in this study there was not an association with infections except for otitis media. The land application of sewage sludge raises the possibilities of such combined expose but there is no hard evidence (*58*).

Metals and metalloids

Metals have been demonstrated to have a range of effects on hosts with respect to infections. Primarily these effects are mediated through altered

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immune system function where acute or chronic exposure to metals or metalloids can augment or suppress an immune response (*59*). There is also considerable evidence that some metals, notably mercury and gold, can give rise to autoimmunity (*60, 61*). Table 1 summarizes documented interactions of metals with bacteria to affect infection of toxicity to the host.

There is evidence for alterations in metabolism that are related to metal concentrations and metals can play a role in modulating stress response. Even metal or metalloid dietary deficiencies can play a role in infections; selenium is needed for proper function of the immune system and its deficiency increases viral pathologies in mice (*82*). Moreover, viral infections in a selenium deficient host can lead to mutations in the virus itself that increase its virulence (*83*).

The effects of heavy metals on infections are not simple. Since they can affect both immune function and individual compounds such as enzymes or receptors by binding at active sites, deliberate or accidental administration can potentially affect both the pathogen and the host. Therefore, the observed result in challenge studies can depend on which is the most affected. This is well illustrated by experiments with Salmon and a flatworm ecto-parasite (*84*) where metals could in some cases be used to control the parasite.

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Negative drug microbe interactions in host

The range of such drug- microbe interactions in the host is potentially vast. In the following examples we make no attempt to exhaustively search the literature, but simply draw attention to some of the more well known examples that are illustrated in Table 2

Once again, it should be emphasized that such interactions are complex and likely not well understood. We make no attempt in this review to document the presence of drugs in the environment; this is an increasing priority for environmental research and is being actively documented in many studies. Environmental exposures, while very much lower in concentration, occur in mixtures. Whether or not such mixtures have human health effects is unknown but since discharge is usually to water sources, it is likely that aquatic organisms would be the 'first responders' and indicators of environmental impacts.

Viruses and chemicals as co-carcinogens

The etiology of cancer is recognized to be complex and potentially multifactorial. Though there is strong evidence for links between cancer and genotoxic chemicals or certain viruses, there is a much more limited body of evidence that suggests a possible synergy between exposures to both these types of agents. Co-carcinogenesis denotes the additive or synergistic effects on cancer development due to multiple agents, for example viruses and chemicals.

Table I. Interactions of metals with the host-pathogen relationship

Metal	*Infectious agent*	*Effect*	*Reference*
Mercury	bacterial endotoxin	potentiates nephrotoxicity (rat)	*62*
Mercury	*Listeria monocytogenes*	decreased body weight and survival; increased infection in organs with chronic exposure (mice)	*63*
Mercury	Bacterial LPS	LPS aggravated mercury-induced autoimmunity	*64*
Cadmium & manganese	Encephalomyocarditis- Semliki Forest -, and Venezuelan Equine Encephalitis viruses	early onset of virus infection and up-regulation of cytokines (mice)	*65*
Cadmium	1 – *Pasteurella multocida* (mice) 2 – Salmonella enteritidis (rats) 3 – influenzavirus A (mice)	airborne challenge raised bacterial mortality but decreased mortality seen with Influenzavirus A compared to Al^{+++} control challenge	*66, 67*
Lead	Semliki Forest virus	enhances virus multiplication and pathogenesis (mice)	*68*
Cadmium, lead, copper, zinc	Cucumber mosaic virus and tomato mosaic virus	affect plant accumulation of heavy metals	*69*
Cobalt, nickel, chromium, titanium, molybdenum	*Staphylococcus epidermidis*	Cobalt, nickel and cobalt-nickel alloy inhibit bacterial phagocytosis by polymorphonuclear leucocytes	*70, 71*
Zinc, copper, cadmium, arsenic	*Ureaplasma urealyticum*	Infection resulted in increase of ratios Cu/Zn and Cd/Zn and concentrations of As and Mg in seminal fluid	*72*
Zinc, cadmium, copper	Infectious pancreatic necrosis virus	Increased mortality (grouper)	*73*

Continued on next page.

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Table I. *Continued.*

Metal	*Infectious agent*	*Effect*	*Reference*
Copper, cyanide. ammonium, nitrite	*Saprolegnia parasitica*	Ammonium and nitrite increased infections; copper or cyanide did not	*74*
Copper, zinc	*Listeria monocytogenes*	Increased or decreased infection (Cu); decreased infection (Zn)	*75*
Copper, zinc	*Brucella abortus*	Serum levels of Cu higher and Zn lower (human)	*76*
Copper	*Aeromonas hydrophila*	Increased susceptibility to infection (catfish)	*77*
Aluminum	*Listeria monocytogenes*	Altered immune response and decreased survival with chronic exposure (mice)	*78*
Arsenic	immune system cells	Predispose to opportunistic infections	*79, 80*
Arsenic	1 - streptococcal aerosol 2 - *Klebsiella pneumoniae*	1 -greater mortality on challenge 2 - reduced pulmonary bactericidal activity	*81*

Table II. Examples of negative drug microbe interactions and host effects

Drug	*Microbe*	*Host Effect*	*Reference*
Infliximab	(Crohn's disease), mycobacteria, *Candida*, *Nocardia*, *Aspergillus*, *Legionella*	Reactivation of latent granulomatous conditions and infections	*85-89*
Aspirin, ibuprofen, etc.	Infections with influenza-, corona-, hepatitis A, & varicella-zoster viruses	Reye's syndrome	*90-93*
Aspirin	Respiratory viral infections	Induced asthma	*92, 94*
Paracetamol	Viruses, mycoplasma, chlamydia in upper respiratory tract infections	Hepato-toxicity	*92, 95*
Methotrexate	Epstein-Barr virus (EBV)	Lymphoma	*96, 97*
antibiotics	*Clostridium difficile* *Candida sp,* *Klebsiella oxytoca*	Diarrhea	*98-101*
Drug pharmacokinetics & toxicity	Viral infections	Altered drug penetration or increased toxicity or both	*102*
Sulphonamides & other drugs	Human immune deficiency virus (HIV)	Hypersensitivity, reaction to drugs and skin conditions	*103, 104*

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There are epidemiological studies that support such interaction in causation of a variety of cancers, and these are summarized in Table 3. In addition, there are a variety of cell culture or animal models where such co-carcinogenesis can be demonstrated (*105*).

If one looks at environments where there is a larger than usual exposure to chemicals and infectious agents, one might think of farms and sewage treatment plants. Evidence from these environments is conflicting, there are some reports of such occupational exposures being associated with increased cancers (*106-109*), but the evidence is not conclusive (*110*). Moreover whether infectious or chemical agents would be the prime candidates can be uncertain. An alternative explanation for environments with relatively large exposures to microorganisms coupled with some exposure to chemical mixtures is that microbial toxins such as bacterial endotoxins or mycotoxins may play a role. Although the role of certain mycotoxins in carcinogenesis seems to be supported, the evidence for endotoxins is much less conclusive with lipid A (*111*) and endotoxins in general (*112*) being possibly protective. However, there is a good reason to consider that bacterial endotoxin may promote the damage caused by other chemicals (*113, 114)* or infectious agents (*115*), and thus may play an indirect role. This is exemplified also be the use of lipid A as an adjuvant in some anticancer therapies (*111*).

The interaction of chemicals and viruses in the host may not always lead to cancer, and there are a number of reports where chemical exposure has led to reactivation of latent virus infections (*116-118*) or virus infection can modify the detoxification of other pollutants (*119*).

Chemical Effects on Microorganisms

Microorganisms are certainly vulnerable to effects from environmental chemicals of all types because of their ubiquity, small size and large surface area to volume ratio. While there are many types of microorganisms, and magnitude of chemical effects may be similar, we will concentrate on bacteria because their behaviour is better understood and documented. For the most part the concentrations of chemicals in natural environments are unlikely to be of sufficient concentration to kill large numbers of bacteria. However, especially with chronic sub-lethal exposure, genotoxic chemicals will cause mutations in bacteria when contact is made that is analogous to that in mammalian cells. Such mutations could kill the cell but more likely they will cause, if anything, a minor change in the cell that will make it more or less fit for its environment, more or less pathogenic for its host(s), etc. Also, though bacteria have no immune system, they do have a variety of stress responses that can be triggered by a wide range of external stimuli. Such responses have evolved to permit the bacteria to make changes on the fly and to adapt rapidly to their environments. These phenotypic changes can sometimes become permanent when mutants arise.

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Table III. Studies implicating Viruses and Chemicals in Co-carcinogenesis

Infectious agent	*Co-carcinogen(s)*	*Cancer*	*References*
Human papillomavirus (HPV) 16, 18, etc.	Tar-based vaginal douches, tobacco Cooking over wood burning stove, HSV-2	Cervical cancer	*120-126*
Hepatitis B virus (HBV)	Dietary aflatoxins	Hepato-cellular carcinoma	*127-129*
HBV/Hepatitis C virus (HCV)	Alcohol, agricultural pesticide use, military service in South Vietnam	Hepato-cellular carcinoma	*130, 131*
Human herpesvirus (HHV) 8	Human immune deficiency virus (HIV), nitrite inhalants	HIV-related Kaposi's sarcoma	*132, 133*
HHV-8	Immuno-suppressants, exposure to water and or volcanic soils, nitrosamines	Kaposi's sarcoma in Uganda	*134-136*
Epstein-Barr virus (HBV)	Malaria, nitrosamines	Burkitt's lymphoma	*137*
EBV	PCBs, pesticides, hair dyes	Non-Hodgkin's lymphoma	*137-139*
EBV	dioxins, dibenzofurans	Non-Hodgkin's lymphoma	*140*
EBV	pesticides, organic solvents, animals, exhausts	Hairy cell leukemia	*141*
HPV	Alcohol, tobacco	Oral cancer	*142, 143*
Simian virus (SV)-40	Asbestos, tobacco	Mesothelioma	*144, 145*
HIV	Tobacco	Lung cancer	*146*

Modified from Haverkos (*147*)

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Bacteria in many natural environments are largely saprophytic and grow under low nutrient conditions, but those pathogens that normally grow on or in human or animal hosts are accustomed to much higher nutrient levels. When these bacteria are present in the environment they are often under starvation stress; intracellular pools of metabolites will decline and many of them may become rather inactive, often going into a viable but non-culturable (VBNC) state where they become difficult to detect by conventional culture. VNBC bacteria could be considered to be 'hibernating'; metabolic activity declines but a significant level of DNA turnover can still be documented. Under these starvation conditions the bacteria are more likely to make replication errors and or be unable to repair damage caused by genotoxic chemicals; the result being mutations that are orders of magnitude greater than those observed for cultures grown under ideal conditions in the laboratory.

It is well recognized that bacteria can acquire through intrinsic adaptation, or by mutation or gene acquisition, a phenotype that is resistant to the prevalent toxic(s), and the best known example is that of antibiotic-resistant bacteria, though many others can be cited. Also, the presence of environmental pollutants can cause a change in the composition of microbial communities. The bacteria most advantaged by the pollutant presence are those able to use it as a nutrient source because they will tend to proliferate while certain other species decline. Next to benefit will be those that can exploit the increased activities of the most advantaged, and so on.

It is quite beyond the scope of this review to examine all the effects of environmental chemicals on microorganisms; nor would we be able to do so because very little is known except for those organisms that can or 'have been trained' to deal with toxic spills or adapted to locales with high levels of metals (*148*). We will, therefore, restrict our discussions to those areas and chemicals which have the most impact on infections. These are the biocidal and preservative chemicals formulated and applied to kill microorganisms or to preserve products and prevent microbial proliferation, respectively.

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The relevant questions

Microbicides are used in food production, in water purification and distribution, in institutional control of infections, in domestic environments for general hygiene and in a variety of industrial uses to prevent fouling and excessive microbial growth. As mentioned above, they are used at very high concentrations and in increasing quantity. The main concerns with their use are:

- their potential genotoxicity for humans and environmental species, including the ability to cause mutations in bacteria;
- whether bacterial exposure to sub-lethal levels will cause resistance to develop, and

- if such resistance develops, whether it can make the bacterium cross resistant to the antibiotics normally administered therapeutically to treat infections.

The argument that resistance to healthcare microbicides at their use dilutions is unlikely to develop is supported by reasoned argument and some studies at high biocide concentrations, but it belies the fact that the use-dilutions of microbicides are not maintained under many conditions of use, nor upon discharge and subsequent dilution in the environment. There is, therefore, exposure of a myriad of bacteria to microbicides at a range of concentrations where lethality can range from none to almost total depending on the species and a variety of other factors.

We would argue that development of resistance to microbicides and the potential for cross-resistance to antibiotics is more likely to occur at very much lower concentrations. The point is purely a statistical one. At high concentrations of a genotoxic biocide there are a) few microorganisms remaining that have the potential to develop resistance and b) the higher numbers of chemical gene interactions are more likely to produce lethal mutations. At lower concentrations, the numbers of surviving bacteria are much higher and so there is a larger pool of individuals with an appropriate response that could become fixed through mutation. Moreover, continuous or repeatedly intermittent exposure to biocides at sublethal levels increases the chances of mutation. In view of the above argument, we maintain that studies that simply look at whether there is a change in the minimum inhibitory concentration (MIC) or minimum biocidal concentration (MBC) are potentially missing the 'event' unless it is done with a very large number of individual isolates. On the other hand, if the level of genotoxic substance is too low then the chemical-gene interactions may be insignificant. What is the appropriate concentration? We consider that the ideal concentration to select would be that on a dose-response curve just before any killing of the microorganism by the biocide is observed. This will give maximum exposure of the maximum population. Careful experimental design is, however, required as confounding with the selection of naturally resistant clonal populations is possible (*149*).

In considering whether resistance can develop, we can look at the range of options for the bacterium exposed to a toxic/genotoxic chemical.

- It can change the permeability of the membrane and keep the chemical out. While this sounds like the ideal solution to repel assaults, it potentially leaves the bacterium at a distinct disadvantage if the same permeability is what it depends on to acquire its nutrients and to multiply.
- It can permit access but actively expel the chemical before it damages the cell. This requires energy expenditure on the part of the bacterium, but such 'efflux pumps' are commonly found in bacteria and can be up-regulated to

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deal with emergency exposure. Resistant mutants might have constitutively-expressed efflux pumps.

- It can detoxify the chemical moiety. This is also likely to be an energy-dependent process and may not be metabolically possible because the end product of a reaction could be more toxic that the original compound.
- It can sequester the chemical in a harmless manner inside the cell by production of 'binding substances' – also energy demanding.
- It can modify the targets that the chemical can interact with. This option is less relevant to microbicides because in contrast to drugs they can generally interact with many targets in the cell. However, trichlosan may be an exception in this regard.
- Even if the bacterium is not equipped to 'resist' the chemical initially, in a naturally mixed population under continuous exposure it may acquire the capability to do so through horizontal gene transfer. Furthermore, growth in a biofilm with substantial protective extracellular polysaccharide matrix may limit access of the chemical to the cell.

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The evidence

In relation to infections, the key question is whether or not acquiring resistance to a preservative or biocide can overlap with resistance to one or more relevant therapeutic antibiotics. What is the evidence?

Well, the best evidence is for trichlosan acting on Gram-negative bacteria; cross-resistance has been observed in a number of studies (*150-155*), and one of the main reasons for this is the specificity of its interaction (*156*). The main mechanism of resistance is through efflux pumps (*157*). However, this is not the only evidence (*157-159*). Laboratory studies have clearly demonstrated the cross-resistance phenomenon, but many believe that it will not occur in the field. We would argue that if it occurs in the laboratory it probably also occurs in the field but much less frequently than in a contrived situation; so studies to demonstrate field occurrence are rather unlikely to pick it up unless they are appropriately designed.

The discussion of a given environmental chemical's effects on microorganisms should not rest entirely on cross-resistance between microbiocides and antibiotics. Exposure to other stressors can up-regulate stress-response genes and affect the susceptibility of bacteria to killing by biocides (*160, 161*). Similar mechanisms may be involved in the decrease in susceptibility to oxidative microbicides after a first exposure. Cross-resistance between pharmacologically active drugs and antibiotics has also been observed (*162*).

Other direct effects have been observed on bacteria by environmental pollutants. For example, exposure to sub-lethal levels of hospital disinfectants can increase sporulation in *Clostridium difficile* (*163*), and exposure to airborne particulates can increase growth in *Haemophilus influenzae* (*164*). Lipophilic environmental pollutants can change cell membrane composition (*165*).

Concluding Remarks

Given the complexity of microbial life forms and the many unknowns that exist, it is perhaps not surprising that concurrent or sequential exposures of susceptible hosts to both chemicals and pathogens is poorly studied, especially with respect to human exposures. Model systems, while useful to explore particular questions rarely reflect real exposure conditions. Moreover, while we know a significant amount about the genetics and molecular biology of our most important pathogens, we know relatively little about their biochemical ecology (*166*) or why they persist as they do in natural or engineered environments, and how they are affected by the chemical, physical and biological composition of their milieux. If, as we believe we have demonstrated, there is significant reason to consider combined interactions of pathogens and pollutants on the susceptible host, then more study of this area is definitely needed. To be relevant and readily interpretable without exaggeration, one should ideally look at the most prominent pathogens and chemical exposure conditions that are as close to reality as possible. This may require inclusion of relevant size particulates.

There are many areas missing from this review but which need exploring in more detail. To have included these would have made the review unwieldy and would require multidisciplinary in-depth knowledge of both authors and readers. That the environment and human infections are complex is an understatement, but insufficient reason for playing ostrich and hoping that the problem will disappear. Research needs are therefore clearly interdisciplinary in nature and comprehensive in scope, with fundamental implications for public health and disease prevention as our exposures to chemicals and pathogens continue to increase in urban societies.

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Chapter 2

Disinfection and Sterilization in Healthcare Facilities

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The effective use of proper disinfection and sterilization procedures plays a significant role in preventing healthcare associated infections. The purpose of this chapter is to describe the scientific rationale for disinfection and sterilization strategies used in hospitals and other healthcare facilities with a focus on chemical germicides formulated as disinfectants, and sterilization procedures that have been developed in the past decade. (move stentence up)Chemical germicides used in healthcare facilities include aldehydes, such as glutaraldehyde, ortho-phthaldehyde (OPA) and formaldehyde, peroxygens, which include hydrogen peroxide, peracetic acid and chlorine dioxide. Medical devices for many years have been sterilized by heat - steam autoclaving and dry heat. There are an increasing number of devices that cannot be sterilized by heat and must be sterilized by low temperature sterilization systems or disinfected by liquid chemical germicides. Ethylene oxides gas, and to a limited extent formaldehyde-steam, have been used for sterilization of heat sensitive instruments. Within the past 10 years a number of new low temperature sterilization systems, including hydrogen peroxide gas plasma sterilizers, and new high level disinfectants have been developed to process medical devices. These systems tend to be oxidative in their mode of action, use relatively low concentrations of the sterilizing agent, do not leave toxic residuals, have excellent compatibility with materials of medical devices, and have short process times.

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The purpose of this chapter is to discuss disinfection and sterilization strategies that are used in health care settings to prevent the transmission of infectious agents from contaminated medical devices and environmental surfaces to patients and health care workers.

The effective use of antiseptics, disinfectants and sterilization procedures in health care settings is important in the prevention of hospital-acquired infections. Historically, the use of physical agents, such as moist heat in the form of steam autoclaves or dry heat sterilizers, has played the predominant role for sterilizing devices, equipment and supplies in hospitals. In the 1990's there were a number of devices developed that were heat sensitive and had to be sterilized by low-temperature sterilization systems. Ethylene oxide was used for many years but concerns about toxicity to humans and the environment and very long sterilization cycles stimulated the development of a number of sterilization procedures meant to replace ethylene oxide sterilization. These include systems that employ hydrogen peroxide gas plasma, ozone, and in some parts of Europe, steam/formaldehyde. Liquid chemical germicides formulated as sterilants have also been available for many years but primarily are used to disinfect rather than sterilize medical devices (1,2,3)

The choice of what sterilization or disinfection procedure or which specific chemical germicide should be used for sterilization, disinfection or antisepsis or for environmental sanitization depends on several factors. No single chemical germicide or sterilization procedure is adequate for all purposes. Factors that should be considered in the selection of a specific sterilization or disinfection procedure include (1) the degree of microbiological inactivation required for the particular device, (2) materials compatibility of the device with the disinfection or sterilization procedure based on the device's nature and physical composition, and (3) the cost and ease of using a particular procedure.

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Regulation of Chemical Germicides

Chemical germicides used as disinfectants or antiseptics in most countries are regulated by the federal or central government and usually by the Public Health Service or Ministry of Health. In the USA, chemical germicides are regulated by two governmental agencies: the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA). Chemical germicides formulated as sterilants or disinfectants had historically been regulated by EPA, but recently the EPA and FDA agreed that sterilants or disinfectants that are used on medical devices (e.g. hemodialysis machines, endoscopes, high-speed dental hand pieces, etc.) will be regulated by FDA. The EPA requires manufacturers of chemical germicides formulated as sanitizers, disinfectants, hospital disinfectants or sterilant/disinfectants (sporicides) to test these products using specific, standardized assay methods for microbicidal potency, stability

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and toxicity to humans. For chemical germicides intended for use on medical devices (as opposed to environmental or housekeeping surfaces), the FDA requires that manufacturers submit a premarket application that may include additional specific microbicidal activity data, device/chemical compatibility data and detailed instructions to the user regarding the `safe and effective use' of the product. FDA also regulates all sterilization devices such as steam autoclaves, ethylene oxide, ozone, and hydrogen peroxide gas plasma sterilizers, and dry heat ovens.

FDA regulates chemical germicides that are formulated as antiseptics, preservatives or drugs to be used on or in the human body or as preparations to be used to inhibit or kill microorganisms on the skin. This type of chemical germicide is categorized basically by use pattern (e.g. antimicrobial handwashes, patient preoperative skin preparations, skin wound cleansers, skin wound protectants and surgical hand scrubs) and is not regulated or registered in the same fashion that EPA regulates and registers a disinfectant. Currently, data are not available to assess accurately the efficacy of many of the antimicrobial antiseptic formulations on the market; consequently, health care workers must make product selection decisions based on information derived from the manufacturer and published studies in the literature.

The Centers for Disease Control and Prevention (CDC) does not approve, regulate or test chemical germicides formulated as disinfectants or antiseptics. Rather, the CDC recommends broad strategies for the use of sterilants, disinfectants and antiseptics to prevent transmission of infections in the health care environment.

Definitions

The definitions of sterilization, disinfection, antisepsis and other related terms such as decontamination and sanitization are generally accepted in the scientific community, but some of these terms are misused. It is important not only to understand the definition and implied capabilities of each procedure, but also to understand how to achieve and in some cases monitor each state.

Sterilization and disinfection

The term *sterilization* is one that students and professionals have memorized and recited seemingly forever. It can be the simplest and the most complex concept depending on how it is viewed and how it is applied. The definition of sterilization can change depending on the user's vantage point. This term can be viewed somewhat like a hologram and can be defined in the context of:

1. The *state* of sterilization,
2. The *procedure* of sterilization,
3. The *application* of sterilization.

Any item, device or solution is considered to be sterile when it is completely free of all living microorganisms. This state of sterility is the objective of the sterilization procedure and, when viewed in this context, the definition is categorical and absolute, i.e. an item is either sterile or it is not.

A sterilization *procedure* is one that kills all microorganisms, including high numbers of bacterial endospores. Sterilization can be accomplished by heat, ethylene oxide gas, hydrogen peroxide gas plasma, ozone, and radiation (in industry). From an operational standpoint, a sterilization procedure cannot be categorically defined. Rather, the procedure is defined as a process, after which the probability of a microorganisms surviving on an item subjected to the sterilization procedures is less than one in one million (10^{-6}). This is referred to as the `sterility assurance level', and it is this approach that is used by the medical device industry to sterilize large quantities of medical devices.

The *application* of sterilization principles in industry is much more sophisticated and controlled than sterilization procedures used in hospitals. However, steam autoclaves, ethylene oxide gas sterilizers, hydrogen peroxide gas plasma sterilizers, and dry heat sterilization ovens used in health care facilities have operational protocols that are verified by the manufacturer to accomplish sterilization, and all the variables that control the inactivation of microorganisms are either automated or built into simple controls in the devices. In addition, the cycles are routinely monitored with chemical and biological indicators.

The *application* of the sterilization process takes into account additional considerations. This approach involves the strategy associated with a particular medical device (or medical fluid) and the context of its degree of contact with patients. Dr. Earl Spaulding in 1972 (1) proposed that instruments, and medical devices be divided into three general categories based on the theoretical risk of infection if the surfaces are contaminated at time of use. Briefly, these categories are: medical instruments or devices that are exposed to normally sterile areas of the body require sterilization; instruments or devices that touch mucous membranes may be either sterilized or disinfected; and instruments, medical equipment or environmental surfaces that touch skin or come into contact with the patient only indirectly can be either cleaned and then disinfected with an intermediate-level disinfectant, sanitized with a low-level disinfectant or simply cleaned with soap and water. These instruments or other medical surfaces are termed `critical', `semi-critical' or `non-critical', respectively. Selection of the appropriate disinfecting procedure in the last category (non-critical will include consideration of the nature of the surface as well as the type and degree of contamination.

In the context of these categorizations, Spaulding also classified chemical germicides by activity level (Table 1):

1. *High-level disinfection.* This is a procedure that kills vegetative microorganisms but not necessarily high numbers of bacterial spores. These

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chemical germicides, by Spaulding's definition, are those that are capable of accomplishing sterilization, that is they kill all microorganisms, including a high number of bacterial spores, when the contact time is relatively long (6-10 h). As high-level disinfectants, they are used for relatively short periods of time (5-30 min). These chemical germicides are very potent sporicides and, in the USA, are those registered with the FDA as sterilant/disinfectants http://www.fda.gov/cdrh/ode/germlab.html (Table II).

2. *Intermediate-level disinfection.* This is a procedure that kills vegetative microorganisms including *Mycobacterium tuberculosis*, all fungi and most viruses. These chemical germicides often correspond to EPA-approved `hospital disinfectants' that are also `tuberculocidal'.

3. *Low-level disinfection.* This is a procedure that kills most vegetative bacteria except *M. tuberculosis*, some fungi and some viruses. These chemical germicides are often ones that are approved in the USA by EPA as hospital disinfectants or sanitizers.

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Spaulding's system for classifying devices and strategies for disinfection and sterilization is quite conservative. There is a direct relationship between the degree of conservatism as expressed by the probability of a microorganism surviving a particular procedure and the microbicidal potency of the physical or chemical germicidal agent. For example, a sterilization procedure accomplished by steam autoclaving, ethylene oxide gas, or hydrogen peroxide gas plasma sterilization, by design and definition, will result in a one-in-one million probability of a surviving microorganism if the procedure had initially been challenged with 10^6 highly resistant bacterial spores. The risk of infection resulting from the use of an item that was subjected to this type of procedure, assuming that the procedure had been carried out properly, would appear to be zero. Correspondingly, the probability of contamination and the theoretical probability of infection associated with sterilization or high-, intermediate- or low-level disinfection with liquid chemical agents would increase as the overall germicidal potency of the selected germicidal agent or procedure decreased.

A process of liquid chemical sterilization would, at best, be three orders of magnitude less reliable than a conventional heat sterilization procedure. From a practical standpoint, this means that there is a lower level of confidence with such procedures, and if and when mistakes are made there is a higher chance of failure than with a heat sterilization procedure. When procedural errors are made, the consequences are magnified especially when a procedure of lower overall potency is used. Procedures that contain fewer built-in assurances are procedurally driven and are ones that should be accompanied by very precise protocols, policies and quality assurance monitoring.

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Table I Activity Levels of Selected Liquid Germicides[a]

Procedure - Chemical	Concentration	Activity level
	STERILIZATION	
Glutaraldehyde	Variable	Not applicable
Hydrogen peroxide	6-30%	
Formaldehyde	6-8%[b]	
Chlorine dioxide	Variable	
Peracetic acid	Variable	
	DISINFECTION	
Glutaraldehyde	Variable	High to intermediate
Ortho-phthalaldehyde	0.5%	High
Hydrogen peroxide	3-6%	High to intermediate
Formaldehyde	1-8%	Intermediate to low
Chlorine dioxide	Variable	High
Peracetic acid	Variable	High
Chlorine compounds[c]	500 to 5000 mg/L free/available chlorine	Intermediate to low
Alcohols (ethyl, isopropyl)[d]	70%	Intermediate
Phenolic compounds	0.5-3%	Intermediate to low
Iodophor compounds[e]	30-50 mg/L free iodine; up to 10,000 mg/L available iodine	Intermediate to low
Quaternary ammonium compounds	0.1-0.2%	Low

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a. This list of chemical germicides centers on generic formulations. A large number of commercial products based on these generic components can be considered for use. Users should ensure that commercial formulations are registered with EPA or FDA.

b. Because of the ongoing controversy of the role of formaldehyde as a potential occupational carcinogen, the use of formaldehyde is limited to certain specific circumstances under carefully controlled conditions, e.g., for the disinfection of certain hemodialysis equipment. There are no FDA cleared liquid chemical sterilant/disinfectants that contain formaldehyde.

c. Generic disinfectants containing chlorine are available in liquid or solid form, e.g., sodium or calcium hypochlorite. Although the indicated concentrations are rapid acting and broad-spectrum (tuberculocidal, bactericidal, fungicidal, and virucidal), no proprietary hypochlorite formulations are formally registered with EPA or cleared by FDA. Common household bleach is an excellent and inexpensive source of sodium hypochlorite. Concentrations between 500 and 1000 mg/L chlorine are appropriate for the vast majority of uses requiring an intermediate level of germicidal activity; higher concentrations are extremely corrosive as well as irritating to personnel, and their use should be limited to situations where there is an excessive amount of organic material or unusually high concentrations of microorganisms (e.g., spills of cultured material in the laboratory).

d. The effectiveness of alcohols as intermediate level germicides is limited, since they evaporate rapidly, resulting in very short contact times, and also lack the ability to penetrate residual organic material. They are rapidly tuberculocidal, bactericidal and fungicidal, but may vary in spectrum of virucidal activity. Items to be disinfected with alcohols should be carefully pre-cleaned and then totally submerged for an appropriate exposure time (e.g., 10 minutes).

e. Only those iodophors registered with EPA as hard-surface disinfectants should be used, and the instructions of the manufacturer regarding proper dilution and product stability should be closely followed. Antiseptic iodophors are not suitable for disinfecting medical instruments or devices or environmental surfaces.

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Table II. FDA-Cleared Sterilants and High Level Disinfectants with General Claims for Processing Reusable Medical and Dental Devices as of May 13, 2005

Manufacturer	Active Ingredient(s)	Sterilant Contact Conditions	High Level Disinfectant Contact Conditions
K041984 Acecide™ High Level Disinfectant and Sterilant			
Minntech Corp	8.3% hydrogen peroxide 7.0% peracetic acid	**Indication for device sterilization.** 5 hrs at 25°C 5 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	5 min at 25°C 5 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes
K041360 Aldahol III High Level Disinfectant			
Healthpoint LTD	3.4% glutaraldehyde 26% isopropanol	**Indication for device sterilization.** 10 hrs at 20° C Contact conditions based on AOAC Sporicidal Activity Test only.	10 min at 20°C 14 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K012889 Banicide® Advanced for Sterilization and High Level Disinfection			
Pascal Company, Inc.	3.5% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 25°C 30 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	45 min at 25°C 30 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.

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K032959 Cidex® OPA Concentrate			
Advanced Sterilization Products, Johnson & Johnson	5.75% *ortho*-phthaldehyde (OPA)	**No indication for device sterilization.** In-Use solution (0.05% OPA) passes the AOAC Sporicidal Activity Test in 32 hrs at 50°C.	5 min at 50°C Single use – diluted by system **For use in the EvoTech Integrated Endoscope Disinfection System only.** Contact conditions established by simulated use testing with endoscopes.
K030004 Cidex® OPA Solution			
Advanced Sterilization Products, Johnson & Johnson	0.55% *ortho*-phthaldehyde	**No indication for device sterilization.** Passes the AOAC Sporicidal Activity Test in 32 hrs at 20°C and 25°C.	**Manual Processing** 12 min at 20°C 14 days Maximum Reuse **Automated Endoscope Reprocessor (AER)** 5 min at 25°C 14 days Maximum Reuse (For processing in an AER only with FDA-cleared capability to maintain solution temperature at 25°C.) Contact conditions established by simulated use testing with endoscopes.
K013280 Sterilox Liquid High Level Disinfectant System			
Sterilox, Technologies, Inc.	Hypochlorite 650-675 ppm Active free chlorine	**No indication for device sterilization**. Passes the Modified AOAC Sporicidal Activity Test in 24 hrs at 25°C.	10 min at 25°C Single use - generated on site Contact conditions established by simulated use testing with endoscopes.

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Table II. *Continued.*

Manufacturer	Active Ingredient(s)	Sterilant Contact Conditions	High Level Disinfectant Contact Conditions
K003087 Sporicidin Sterilizing and Disinfecting Solution			
Sporicidin International	1.12% glutaraldehyde 1.93% phenol/phenate	**Indication for device sterilization.** 12 hrs at 25°C 14 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	20 min at 25°C 14 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K993042 Rapicide™ High Level Disinfectant and Sterilant			
MediVators, Inc.	2.5% glutaraldehyde	**Indication for device sterilization.** 7 hrs 40 min at 35°C 28 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes and additional supporting information.	**Automated Endoscope Reprocessor** 5.0 min at 35°C 28 days Maximum Reuse (For processing in an AER only with FDA-cleared capability to maintain the solution temperature at 35°C.) Contact conditions established by simulated use testing with endoscopes.
K991487 Cidex®OPA Solution High Level Disinfectant			
Advanced Sterilization Products, Johnson & Johnson	0.55% *ortho*-phthaldehyde	**No indication for device sterilization.** Passes the AOAC Sporicidal Activity Test in 32 hrs at 20°C.	12 min at 20°C 14 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K974188 Cetylcide-G® Concentrate and Diluent Concentrate			
Cetylite Industries, Inc.	3.2% glutaraldehyde	**Indication for device sterilization.**	40 min at 20°C

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		10 hrs at 20°C 28 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	28 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K974062 MedSci 3% Glutaraldehyde			
MedSci, Inc.	3% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 25°C 28 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	25 min at 25°C 28 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K972708 EndoSpor™ Plus Sterilizing and Disinfecting Solution *Note: Due to the lack of test strips for monitoring the concentrations of the active ingredients, the reuse period is limited to 14 days.*			
Cottrell Limited	7.35% hydrogen peroxide 0.23% peracetic acid	**Indication for device sterilization.** 180 min at 20°C 14 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.	15 min at 20°C 14 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K970230 Sporox™ Sterilizing & Disinfection Solution			
Reckitt & Colman Inc.	7.5% hydrogen peroxide	**Indication for device sterilization.** 6 hrs at 20°C 21 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	30 min at 20°C 21 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.

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Table II. *Continued.*

Manufacturer	Active Ingredient(s)	Sterilant Contact Conditions	High Level Disinfectant Contact Conditions
K960513 Peract™ 20 Liquid Sterilant/Disinfectant			
Minntech Corporation	1.0% hydrogen peroxide 0.08% peracetic acid	**Indication for device sterilization.** 8 hrs at 20°C 14 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.	25 min at 20°C 14 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K932922 Procide® 14 N.S.			
Cottrell Limited	2.4% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 20°C 14 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	45 min at 20°C 14 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K932922 Omnicide™ Long Life Activated Dialdehyde Solution			
Cottrell Limited	2.4% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 20°C 28 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	45 min at 20°C 28 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K932922 Omnicide™ Plus			
Cottrell Limited	3.4% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 20°C 28 days Maximum Reuse Contact conditions based on AOAC	45 min at 20°C 28 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.

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		Sporicidal Activity Test only.	
K931592 Metricide Plus 30® Long-Life Activated Dialdehyde Solution			
Metrex Research, Inc.	3.4% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 25°C 28 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only. FDA accepted Metricide Plus as identical to Cidex Plus.	90 min at 25°C 28 days Maximum Reuse FDA accepted Metricide Plus as identical to Cidex Plus.
K931052 Metricide® 28 Long-Life Activated Dialdehyde Solution			
Metrex Research, Inc.	2.5% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 25°C 28 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only. FDA accepted Metricide 28 as identical to Cidex Formula 7.	90 min at 25°C 28 days Maximum Reuse FDA accepted Metricide 28 as identical to Cidex Formula 7.
K930284 Metricide® Activated Dialdehyde Solution			
Metrex Research, Inc.	2.6% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 25°C 14 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only. FDA accepted Metricide as identical to Cidex.	45 min at 25°C 14 days Maximum Reuse FDA accepted Metricide as identical to Cidex.

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Table II. *Continued.*

Manufacturer	Active Ingredient(s)	Sterilant Contact Conditions	High Level Disinfectant Contact Conditions
K924434 Cidex™ Activated Dialdehyde Solution			
Advanced Sterilization Products, Johnson & Johnson	2.4% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 25°C 14 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	45 min at 25°C 14 days Maximum Reuse Contact conditions based on literature references.
K924334 Cidex Formula 7™ Long-Life Activated Dialdehyde Solution			
Advanced Sterilization Products, Johnson & Johnson	2.5% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 20-25°C 28 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	90 min at 25°C 28 days Maximum Reuse Contact conditions based on literature references.
K923744 Cidex Plus™ 28 Day Solution			
Advanced Sterilization Products, Johnson & Johnson	3.4% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 20-25°C 28 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	20 min at 25°C 28 days Maximum Reuse Contact conditions based on literature references.

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K914749 Wavicide® - 01			
Wave Energy Systems	2.5% glutaraldehyde	**Indication for device sterilization.** 10 hrs at 22°C 30 days Maximum Reuse Contact conditions based on AOAC Sporicidal Activity Test only.	45 min at 22°C 30 days Maximum Reuse Contact conditions established by simulated use testing with endoscopes.
K875280 STERIS 20™ Sterilant *Note: Cleared for use with the STERIS System 1™ Processor only.*			
STERIS® Corporation	0.2% peracetic acid	**Indication for device sterilization.** 12 min at 50-56°C Single use only. Contact conditions established by simulated use testing with endoscopes and passing a modified AOAC Sporicidal Activity Test.	**No indication for high level disinfection.**

Decontamination

Another term often used in health care facilities is *decontamination.* A process of decontamination is one that renders a device, items or materials safe to handle, i.e. safe in the context of being reasonably free from disease transmission risk. In many instances, this process is a sterilization procedure such as steam autoclaving, and this is often the most cost-effective way of decontaminating a device or an item. Conversely, the decontamination process may be ordinary soap and water cleaning of an instrument, a device, or an area. When chemical germicides are used for decontamination, they can range in activity from sterilant/disinfectants, which may be used to decontaminate spills of cultured or concentrated infectious agents in research or clinical laboratories, to low-level disinfectants or sanitizers when general housekeeping of environmental surfaces are involved.

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Antiseptic

The term *antiseptic* is used to describe a substance that has antimicrobial activity and is formulated to be used on or in living tissue to remove, inhibit growth of, or inactivate microorganisms. Quite often the distinction between an antiseptic and a disinfectant is not made. However, the differences between a disinfectant and an antiseptic are very great and applications are significantly different. A disinfectant is a chemical germicide formulated to be used solely on inanimate surfaces such as medical instruments or environmental surfaces, whereas an antiseptic is formulated to be used solely on or in living tissues. Some chemical agents such as iodophors can be used as active ingredients in chemical germicides that are formulated either as disinfectants or antiseptics. However, the precise formulations are significantly different, use patterns are different and the germicidal efficacy of each formulation differs substantially. Consequently, disinfectants should never be used as antiseptics and antiseptics should never be used to disinfect instruments or environmental surfaces.

Factors that Influence Germicidal Activity

Microorganisms vary widely in their resistance to sterilants and disinfectants. The most resistant microorganisms are bacterial endospores and few, if any, other microorganisms approach the broad resistance of these organisms (This sentence is confusing and may need modification). A number of factors, some of which are associated with microorganisms themselves and others with the surrounding physical and chemical environment, can significantly influence the antimicrobial efficacy of chemical germicides.

Some factors are more important than others, but all of them should be considered when planning sterilization and disinfection strategies for medical and surgical devices and materials. Briefly, these factors are as follows.

Type of Microorganism

Bacterial spores are more resistant than mycobacteria, fungi, vegetative bacteria and viruses. Some types of viruses are more resistant to germicides than others. As a general guide, one should define the state or degree of inactivation needed (i.e. sterilization or various levels of disinfection) and then choose the most appropriate procedure.

Number of Microorganisms

All other factors being equal, the greater the number of microorganism on a device, the longer it takes to reduce the microbial population. It is for this reason that devices, especially those that are disinfected, should be cleaned prior to being sterilized or disinfected.

Intrinsic Resistance of Microorganisms

Although bacterial spores are more resistant to germicides , very few species in the genera Bacillus or Clostridium are actually responsible for hospital-acquired infections. However, organisms such as *M. tuberculosis var. bovis* and non-tuberculous mycobacteria, as well as naturally occurring Gram-negative water bacteria such as *Pseudomonas aeruginosa* and other pseudomonads can, under some circumstances, be relatively resistant to chemical disinfectants. Second to bacterial spores, *Mycobacterium* spp. is considered one of the most resistant classes of microorganisms to germicides. It is for this reason that chemical germicides that have been approved as "tuberculocides" are sometimes recommended for decontamination or disinfection when a higher activity germicide is needed. It is usually not a concern for transmission of *M. tuberculosis* but rather a definition or specification that can be used to describe a germicide with a relatively broad range of germicidal activity. Resistance of certain non-lipid viruses is similar to that of mycobacteria (Table III) (4).

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TABLE III. Levels of disinfectant action according to type of microorganism

		Bacteria		**Fungi**[1]	**Virus**	
Level of action	Spores	Mycobacteria	Vegetative cells		Non-lipid, small	Lipid, medium sized
HIGH	+[2,3]	+	+	+	+	+
INTERMEDIATE[4]	-	+	+	+	+/-[5]	+
LOW	-	-	+	+/-	+/-	+

1. Includes asexual spores but not necessarily chlamydospores or sexual spores.
2. Plus sign indicates that a killing effect can be expected; a negative sign indicates little or no killing effect.
3. Only with extended exposure times are high-level disinfectants capable of killing high numbers of bacterial spores in laboratory tests; they are, however, capable of sporicidal activity.
4. Some intermediate-level disinfectants (e.g., hypochlorites may exhibit some sporicidal activity; others (e.g., alcohols or phenolics) have no demonstrated sporicidal activity.
5. Some intermediate-level disinfectants, although tuberculocidal, may have limited virucidal activity.

Amount of Organic Soil Present on the Item to be Disinfected or Sterilized

Blood, feces or other organic soil may contribute to failure of a disinfecting or sterilizing procedure in three ways. Organic soil may contain large and diverse microbial populations, may prevent penetration of germicidal agents or may directly inactivate certain germicidal chemicals. This factor, perhaps even more than others, underscores the necessity of pre-cleaning items thoroughly prior to disinfection or sterilization.

Type and Concentration of Germicide

Generally, with all other factors being constant, the higher the concentration of a germicide, the greater is its effectiveness and the exposure time necessary for disinfection or sterilization can be shorter. If a chemical agent is reused over a period of time, the product effectiveness may be reduced due to a variety of factors such as dilution or organic contamination.

Time and Temperature of Exposure

With few exceptions, the longer the exposure times to a given chemical agent, the greater is its effectiveness. An increase in temperature will significantly increase germicidal effectiveness, but deterioration or evaporation of the agent along with an increase in corrosiveness may also occur.

Other Product- or Process-Related Factors

The presence of organic or inorganic loads, pH and the degree of hydration of biological material may significantly affect the potency of certain chemical germicides. For these as well as other factors given above, care should be taken to examine closely and follow label instructions of proprietary germicides.

Device-Related Factors

The device or item being disinfected or sterilized must be physically and chemically compatible with the chosen procedure to ensure effectiveness and continued function of the device or item. Also, factors such as ease of access and cleaning as well as the size of the device or item are important considerations. The manufacturer of the item being reprocessed is the best source of pertinent information in this regard.

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Summary

The choice of specific disinfectants in association with protocols for cleaning is a decision that is made broadly in heath care facilities. No single chemical germicide procedure is adequate for all disinfection or sterilization purposes, and the realistic use of chemical germicides depends on a number of factors that should be considered in selecting available procedures. These include the degree of microbial killing required; the nature and composition of the surface item or device to be treated; and the cost, safety, and ease of use of the available agents.

Strategies for the use of liquid chemical germicides are based on the original suggestions of Dr. Earl Spaulding and consist of two parts. One divides medical devices into categories based on use and risk of infection. The other recommends procedures of (1) sterilization and disinfection: **critical device** (sees blood or tissue), sterilization; (2) **semicritical device** (touches mucus membranes), sterilization or at least high-level disinfection; and (3) **noncritical devices or surfaces** (touches skin or involves environmental surfaces), intermediate or low level disinfection, or detergent cleaning.

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Chapter 3

Disinfection of Prions

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Prions are unprecedented transmissible pathogenic agents that cause a group of fatal neurodegenerative diseases, including Creutzfeldt-Jakob disease (CJD) and kuru in humans, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk, and scrapie in sheep and goats. Prions are resistant to standard disinfection and sterilization procedures validated against viruses and bacteria. There are well documented cases of iatrogenic prion transmission from surgical instruments and cadaveric tissue. The lack of noncorrosive procedures that inactivate prions is a cause for concern for hospital infection-control departments. A novel method to inactivate human prions completely, using sodium dodecyl sulfate at acidic pH (acidic SDS), is reported here. Prion inactivation was demonstrated on both brain homogenate and on the surface of contaminated surgical stainless steel, the latter of which proved significantly more resistant to inactivation.

In New Biocides Development; Zhu, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Prion diseases represent a novel paradigm of infection since the causative agent is devoid of a nucleic acid genome, and is composed solely of an abnormal conformational isoform of the host prion protein (PrP). The abnormal isoform, termed PrP^{Sc} (after scrapie, the prototypic prion disease), serves as a template to recruit molecules of the normal cellular isoform (PrP^{C}) to replicate its misfolded conformation. Prion diseases are therefore disorders of protein conformation involving template-assisted replication, resulting in PrP^{Sc} accumulation in the brain, which invariably causes neuronal dysfunction, degeneration, and death.

The most remarkable feature that distinguishes prion diseases from viral, bacterial, fungal, and parasitic disorders is that prion diseases can manifest as infectious, inherited, or sporadic illnesses. In all cases, infectious prions composed of PrP^{Sc} molecules are generated in the brains of afflicted individuals.

When prions are passaged into the brain of a different host species, a "transmission barrier," related primarily, but not exclusively, to differences in PrP sequence, is responsible for inefficient infection [1-3]. The most notorious example of cross-species transmission is the appearance of variant (v) CJD, in teenagers and young adults in the United Kingdom, from consumption of beef infected with BSE prions [4]. In the USA, there is growing concern that a similar epidemic could occur from the rapid and continuing spread of CWD in both wild and farmed deer and elk, and the possible transmission to humans [5].

A basic understanding of prion biology is essential to the establishment of sound, intelligent, and effective biosafety principles and measures. If prion-related risk is evaluated from the perspective of the virologist, ignoring the unique properties of prions, uninformed and perhaps harmful decisions, based on erroneous assumptions, may result.

Resistance to inactivation

The unusual stability of the infectious agent responsible for scrapie in sheep was first noticed in the 1930s. A significant proportion of sheep inoculated against looping-ill virus, with a specific batch of inoculum, began to develop scrapie approximately two years later. The inoculum was prepared from pooled sheep brains, spinal cords, and spleens, treated with formalin and certified free of any detectable living virus [6]. Concurrently, transmission of sheep scrapie to goats was experimentally demonstrated by inracerebral inoculation of scrapie-infected CNS tissue [7]. It was therefore concluded that the batch of looping-ill inoculum contained tissue from sheep with pre-symptomatic scrapie, and that the infectious agent was unusually resistant to inactivation [6]. Further experiments on the resistance of the scrapie agent to both ionizing and ultraviolet radiation only served to deepen its mysterious nature [8, 9].

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The differences in the inactivation profiles of prions and viruses provided the first clues that the scrapie agent was not a slow virus as had been widely thought [10, 11]. As preparations from Syrian hamster brain were progressively enriched for scrapie infectivity, procedures that modified proteins were found to inactivate the samples, while those that modified nucleic acids had no effect. The results of numerous studies designed to probe the molecular nature of the scrapie agent and define conditions for inactivation concluded that protein denaturants were effective at reducing infectivity titers [12] but that complete inactivation required extremely harsh conditions [13, 14].

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Recommendations for prion inactivation

Currently recommended protocols for prion decontamination include either: (i) >2% available chlorine of sodium hypochlorite for 2 h; (ii) 2 N sodium hydroxide for 1 h; or (iii) autoclaving at 134 °C for 4.5 h [14-18]. Both sodium hypochlorite and sodium hydroxide are corrosive at the concentrations required to inactivate prions and have been reported to inactivate CJD prions incompletely under some conditions [19, 20]. Autoclaving is time-consuming, cannot be used with heat-sensitive or complex materials, and generates dried macerated tissue, in which prions are likely to be more resistant to inactivation. Moreover, these recommendations are largely based on the inactivation of rodent prions in crude brain suspensions. As described below, human prions are substantially more resistant to inactivation than hamster prions. Additionally, prions bound to stainless steel are more resistant to inactivation than those in tissue suspensions. Because of the foregoing limitations, surgical or dental equipment used in operations on patients suspected to have CJD is generally discarded.

Iatrogenic transmission

The need for a noncorrosive denaturant for prions is clearly illustrated by the cases of iatrogenic CJD in which prion-contaminated neurosurgical equipment seems to have spread prions from one patient to another [21-24]. This concern has been heightened in Britain, where the finding of high titers of vCJD prions in lymphoid tissues worries authorities that any surgical procedure could result in the spread of prions from one patient to another [23, 25]. The case of a 69-year-old man with vCJD, who received a blood transfusion from a young donor who eventually died of vCJD, suggests that the transfused blood contained vCJD prions [26]. Subsequently, a second older patient who also received a blood transfusion from a young donor who died of vCJD was reported [27]. While this patient died before developing clinical signs of vCJD, the patient did have prions in his lymphoid tissues. A third case of iatrogenic transmission from transfusion was recently reported, although the complete details have not yet been released [28].

These cases accentuate the urgent need for an effective protocol to inactivate prions as well as to identify prion-infected specimens before they are given as therapeutics as in the case of blood [29]. A recent study of ~12,000 appendices taken at routine operation found three contained PrP^{Sc} based on immunohistochemical (IHC) analyses [30]. The authors argued that this finding suggests as many as 4,000 people in the UK may be carrying prions in their lymphoid tissues. Our recent study comparing the efficacy of IHC to the conformation-dependent immunoassay (CDI) suggests that IHC may underestimate prion disease by a factor of 5 [31]. Whether the number of people in the UK thought to be harboring prions in their lymphoid tissues is closer to 20,000 is unknown. A practical method for routine prion decontamination of all surgical instruments is urgently needed to prevent cases of iatrogenic CJD.

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Prion inactivation studies

Dendrimers and SDS

In the course of studies on the expression of foreign PrP genes in prion-infected cultured cells, branched polyamines, or "dendrimers," were shown to render PrP^{Sc} susceptible to proteolytic degradation [32]. This enhanced susceptibility to degradation could be mimicked *in vitro* by incubating prions at pH ~3.5 with polyamine dendrimers [33]. Intrigued by the ability of weak acids such as acetic acid (AcOH), in combination with dendrimers, to render prions susceptible to proteolytic degradation, we decided to explore the effect of a variety of protein denaturants on prion stability under weakly acidic conditions. Of all the detergents and chaotropes examined, SDS combined with AcOH proved to be the most potent reagent for inactivation of prions. This observation was unexpected since SDS at neutral pH exhibits only a modest ability to inactivate prions in our experience [34]. The experiences of others are also noteworthy: 3% SDS at neutral pH is reported to have destroyed prion infectivity in brain homogenates when samples were boiled or autoclaved [35-37]. However, prion infectivity in macerated brain samples survived boiling for 15 min in 5% SDS at neutral pH [38]. These findings suggest that SDS solutions at neutral pH, even when exposed to high temperatures, cannot be used for complete inactivation of prion infectivity.

Selection of prion strain

Our initial inactivation studies were performed on the Sc237 prion strain in Syrian hamster (SHa) brain homogenates for three reasons: (1) SHa prions are the most well characterized with respect to physical properties; (2) the titers of prions in brain are 10- to 1,000-fold higher than those found in other species; and (3) high-titer samples produce disease in 70 days in hamsters and 45 days in Tg mice overexpressing SHaPrP. These high titers of prions create a broad dynamic range over which measurements can be made and the short incubation times create the most sensitive system available for evaluating low levels of infectivity.

Acidic SDS denatures PrP^{Sc}

To examine whether solutions of 1 to 4% SDS could denature PrP^{Sc} more effectively at different pH values, 50 mM sodium acetate and Tris acetate buffers were used to maintain the pH between 3.5 and 10.0 for homogenates prepared from the brains of Syrian hamsters infected with Sc237 prions. Our studies demonstrated that aqueous solutions of $\geq 1\%$ SDS could denature PrP^{Sc} completely at pH values ≤ 4.5 or ≥ 10.0, as judged by immunoblotting for PrP after the samples were subjected to limited proteolysis (**Figure 1**). Acidic solutions other than AcOH were found to enable SDS to denature PrP^{Sc} in Sc237-infected brain homogenate [39]. However the type of detergent required was much more specific: upon testing a wide range of different detergents, only alkyl sulfates with alkyl chains of 9 to 12 carbon atoms and alkyl sulfonates with alkyl chains of 10 to 13 carbon atoms were effective at denaturing prions [39].

Measuring prion inactivation by bioassay

To determine whether denaturation of PrP^{Sc} caused by acidic SDS correlates with a reduction in prion infectivity, Sc237-infected brain homogenates were incubated for 2 h with different buffers at various temperatures and inoculated intracerebrally into Syrian hamsters. Inoculation of an infectious brain homogenate at neutral pH without detergent caused disease in all animals in 84 ± 0.4 d. Animals inoculated with samples exposed to either 0.5% AcOH or 1% SDS alone for 2 h at room temperature developed disease with similar incubation periods. In contrast, exposure of the inoculum to a solution of 1% SDS and 0.5% AcOH for 2 h at room temperature prolonged the median incubation time to 200 ± 2.3 d [39].

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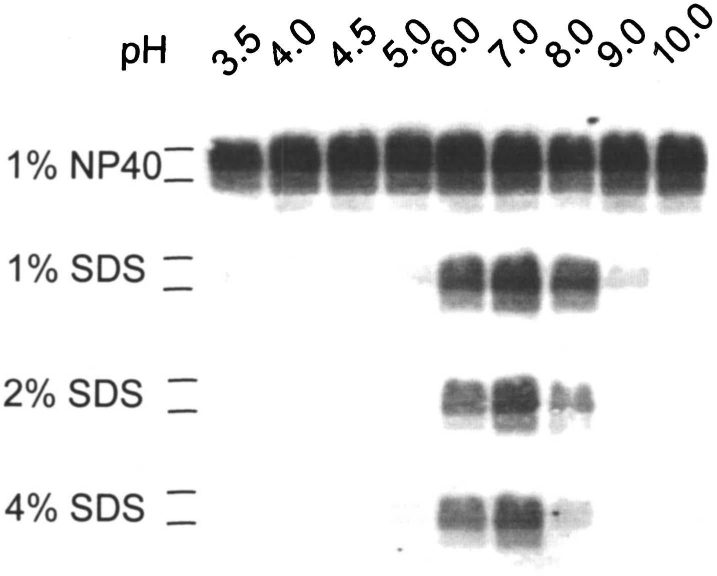

Figure 1. Western blot of prion-infected brain homogenate treated with detergents at various pH. Samples of 1% hamster brain homogenate containing Sc237 prions were incubated for 15 min at 37°C with the indicated detergent and concentration at the indicated pH. Molecular masses based on migration of protein standards are 30 kDa (top) and 27 kDa (bottom).

The prolonged incubation times found after acidic SDS treatment of hamster brain homogenates indicate that the prion titers were reduced by a factor of ~10^7, based on standard curves that relate the incubation time to the dose of prions in the inoculum [40, 41]. However, since all of the hamsters used for bioassay eventually developed disease, the prions were not completely inactivated. Although the data argue that SDS and dilute acid act synergistically to diminish prion infectivity, complete inactivation necessitated modification of the protocol [39].

Transgenic mice in prion research

While these initial studies proved promising, concerns remained that extensive bioassays in hamsters would be complicated by the relatively short lifespan of these animals [42]; therefore transgenic (Tg) mice expressing high levels of SHaPrP, designated Tg7 mice, were employed for subsequent studies [2, 43]. To make these studies more clinically relevant, crude brain homogenates, rather than homogenates pre-cleared by low-speed centrifugation, were used.

A 10% (w/v) Sc237-infected brain homogenate was serially diluted, and each dilution was inoculated into Tg7 mice. Kaplan-Meier analysis was applied to the data to determine the relationship between titer and incubation period (**Figure 2A**). As the infected brain homogenate is diluted, the median incubation period lengthens and the proportion of mice remaining disease-free increases.

Acidic SDS at elevated temperatures

In an attempt to destroy the residual prion infectivity found by bioassay in Syrian hamsters [39], concentrations of SDS were increased to 2% and AcOH to 1%, and the treatment temperature raised to 65°C. Inoculation of a control Sc237-infected brain homogenate at neutral pH without detergent produced disease in Tg7 mice with a median incubation time of 46 d (**Table 1**). Although uninoculated Tg7 mice remained healthy for 500 d, some animals can develop a non-transmissible neuromyopathy after this time [44]. Since mice are inoculated at 8–10 weeks of age, bioassays in Tg7 mice were terminated after 400 days to exclude the possibility of illness not caused by prions. After exposure of Sc237-infected brain homogenates to 2% SDS and 1% AcOH at 65°C for 30 min, the inoculum produced prion disease in some of the mice, with significantly prolonged incubation times. Increasing the exposure time of the inoculum to 2 h or 18 h resulted in all animals remaining disease-free 400 days after inoculation.

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Table 1. Inactivation of prions by 2% SDS + 1% AcOH at 65°C

	Hamster Sc237 prions in Tg7 mice				*Human sCJD prions in Tg23372 mice*			
	Homogenate		Wire		Homogenate		Wire	
	IP	Prop.	IP	Prop.	IP	Prop.	IP	Prop.
Negative control	> 400	1.00	> 400	1.00	> 500	1.00	> 500	1.00
Positive control	46 ± 0.2	0.00	52 ± 0.3	0.00	131 ± 0.7	0.00	215 ± 0.9	0.00
2% SDS 1% AcOH, 30 min	> 400	0.74	82 ± 0.7	0.00	266 ± 8.5	0.26	354 ± 1.6	0.14
2% SDS 1% AcOH, 2 h	> 400	1.00	269 ± 3.2	0.32	> 500	0.74	> 500	0.56
2% SDS 1% AcOH, 18 h	> 400	1.00	> 400	1.00	> 500	0.75	> 500	0.75

Median incubation period (IP) ± standard error, in days, and proportion of animals remaining disease-free (Prop.) were calculated using Kaplan-Meier analysis.

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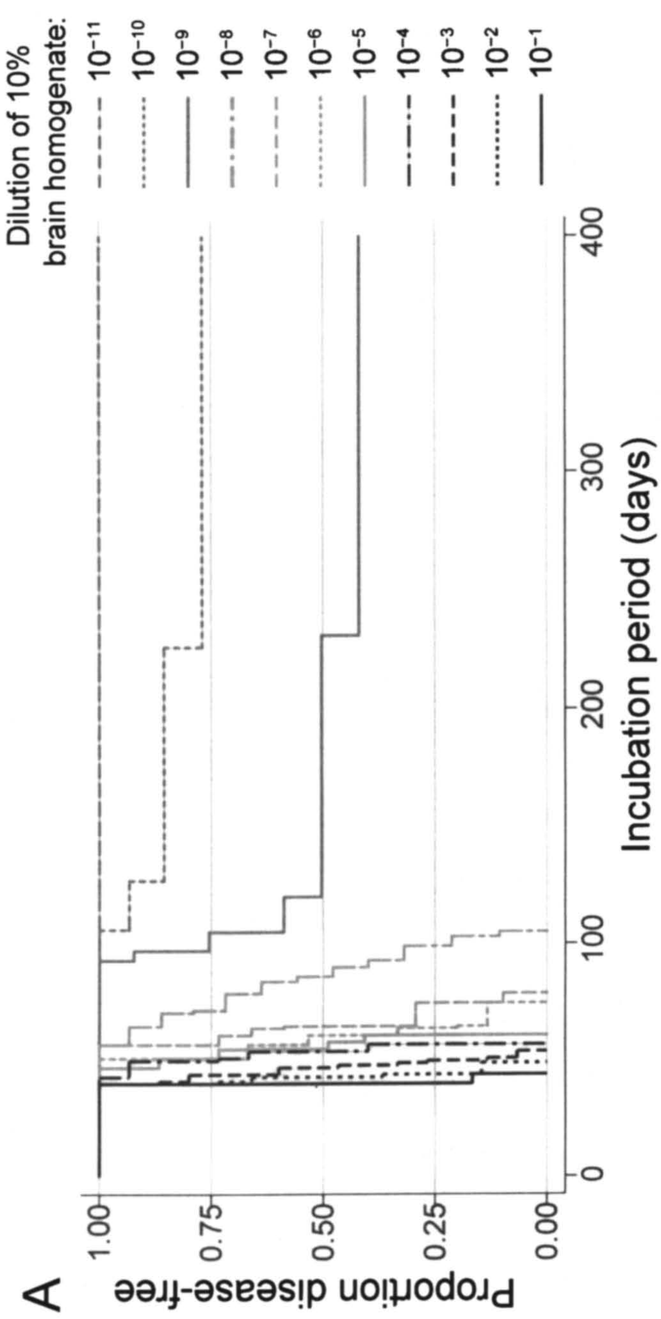

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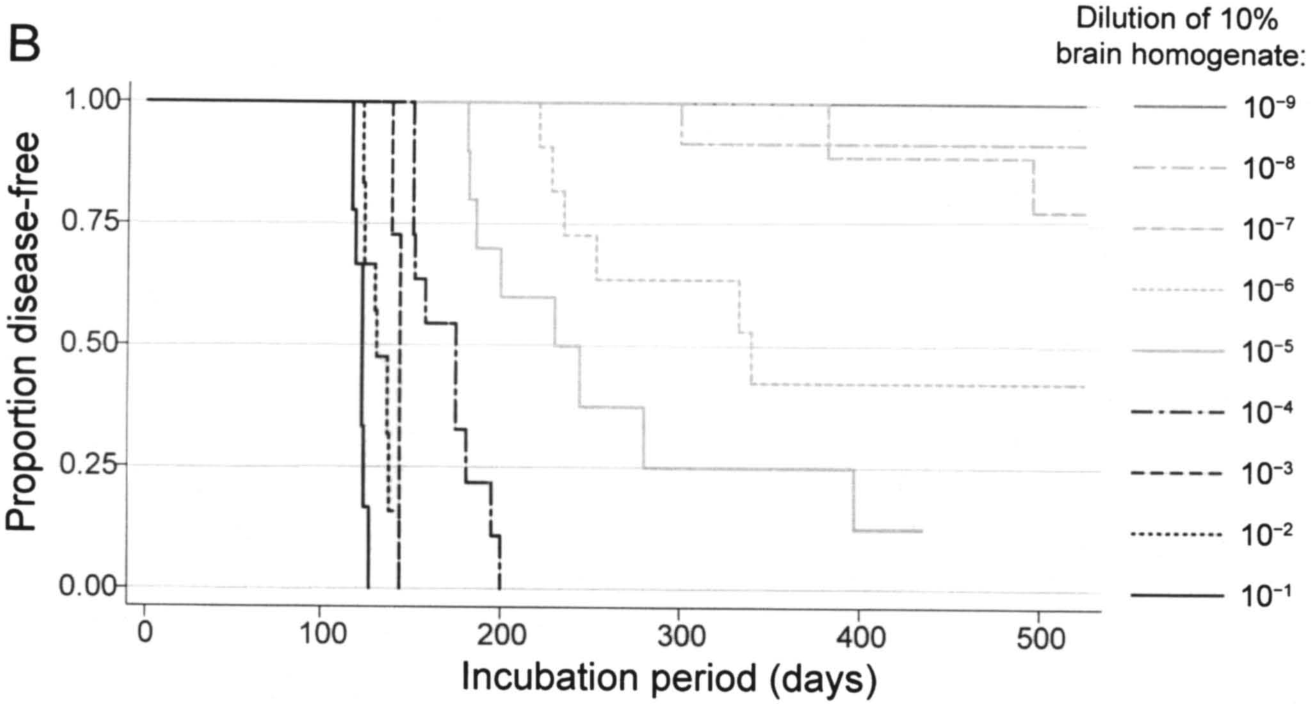

Figure 2. Kaplan-Meier survival curves for 10-fold serially diluted prion-infected brain homogenate. Combined data of three independent experiments for each dilution, each inoculated into five (Sc237) or four (sCJD) mice. (A) Sc237 prions bioassayed in Tg7 mice. (B) sCJD prions bioassayed in Tg22372 mice.

Inactivation of human prions by acidic SDS

Because different prion strains exhibit distinct resistances to inactivation by chaotropic salts and heat [45-49], the inactivation of sporadic (s) CJD prions by acidic SDS was investigated. Brain homogenates were prepared from an sCJD patient homozygous for methionine at polymorphic residue 129, who carried no mutations in the *PRNP* gene, and whose unglycosylated proteinase K (PK)-resistant band migrated at 21 kDa on SDS-PAGE [50]. These human prions, designated sCJD(MM1), is the most common form of CJD [50, 51]. To ensure the highest degree of sensitivity, a line of Tg mice expressing a chimeric human/mouse PrP gene, denoted Tg22372, were utilized; these mice are the most susceptible line for human sCJD(MM1) prions [52]. It is noteworthy that like the human PrP^{Sc} inoculum, the PrP transgene in Tg22372 mice encodes methionine at position 129. Serially diluted brain homogenate containing sCJD(MM1) prions were inoculated into these mice and a similar relationship was deduced between the length of the incubation time and the size of the inoculum dose (**Figure 2B**). Prion disinfection studies were conducted on both intracerebrally inoculated brain homogenate and implanted wires that had been coated with the homogenate.

Sporadic CJD(MM1) prions in brain homogenates were treated with 2% SDS and 1% AcOH at 65°C (**Table 1**). Acidic SDS treatment for 30 min at 65°C prolonged the incubation times from 131 ± 0.7 d to 266 ± 8.5 d. Increasing the time of exposure (2 h or 18 h) to acidic SDS at 65°C lengthened the incubation time and increased the proportion of Tg mice remaining disease-free.

Acidic SDS inactivates prions bound to steel wire

To study the inactivation of prions on surfaces, stainless steel wires were soaked in 10% brain homogenates containing Sc237 or sCJD prions for 16 h at room temperature. The infectivity before and after disinfection procedures was demonstrated by subsequent implantation of the wire into the brain of a reporter animal [53]. This system has become a model of choice to assess the inactivation of prions [39, 54-56].

Wires coated with Sc237 prions were implanted into the parietal lobes of Tg7 mice and produced disease in 52 ± 0.3 d. Treatment of the wires at 65°C for 30 min with 2% SDS and 1% AcOH increased the incubation time to 82 ± 0.7 d after implantation; treatment at 65°C for 2 h further lengthened the incubation time, but most of the Tg7 mice still developed disease in fewer than 400 d. Only treatment at 65°C for 18 h resulted in all animals remaining disease-free at 400 days (**Table 1**). Similarly inactivation of prions from sCJD-coated steel wires resulted in prolonged incubation times, but significant infectivity remained (**Table 1**).

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Acidic SDS and autoclaving abolish prion infectivity

While exposure of Sc237 and sCJD(MM1) prions to 2% SDS and 1% AcOH at 65°C for 2 h was sufficient to destroy more than 99.99% of the infectivity in brain homogenates, complete inactivation was not achieved (**Table 1**). To determine if autoclaving for a brief time in the presence of acidic SDS could eliminate prion infectivity, Sc237 and sCJD(MM1) prions in brain homogenates and on wire surfaces were exposed to 121°C in the presence and absence of acidic SDS (**Table 2**). Neither Sc237 nor sCJD(MM1) prions in brain homogenates or on wire surfaces were detectable by bioassay in Tg mice after exposure to 121°C for 15 min in the presence of 2% SDS and 1% AcOH (**Table 2**).

To quantify the reduction in prion titer from these procedures, Cox proportional-hazards model based on the serial dilution data (**Figure 2**) was employed to derive partial-likelihood ratios based 95% confidence intervals. Treatment of sCJD prions in brain homogenate for 30 min at 121°C (**Table 2**) results in a $10^{6.8}$ reduction (lower 95% confidence interval, $10^{5.3}$). The incubation periods for prion-coated wires cannot be converted into titers since the prions seem to be bound tightly to the surface of the wire [57] and it is not possible to remove them for measurement.

Comparison of human and hamster prions

Exposure of prion infected brain homogenates to acidic SDS resulted in large differences in the level of inactivation for the two prion strains studied (**Tables 1** and **2**; **Figure 3**). Because the titer of Sc237 prions is ~1000-fold higher than sCJD prions in brain homogenates, data were analyzed by a stratified Cox regression [58] in order to quantify differences between hamster and human prions. Taking the ratios of coefficients in the Cox model, it is possible to relate the effect of an inactivation procedure to an approximately equivalent dilution. Applying the stratified Cox regression to the 30-min exposure to acidic SDS at 65°C, a $10^{9.0}$ reduction in infectivity for Sc237 prions and a $10^{3.8}$ reduction for sCJD prions were estimated. The difference in inactivation between the two prion strains is $10^{5.2}$ (95% confidence intervals, $10^{3.7}$–$10^{6.8}$). This analysis argues that sCJD prions in human brain homogenates are more than 100,000 times more resistant to inactivation than Sc237 prions in SHa brain homogenates.

Stabilities of various prion strains

Prion strains, distinguished by incubation times, distribution of neuronal vacuolation, and patterns of PrP^{Sc} deposition, are enciphered in the conformation

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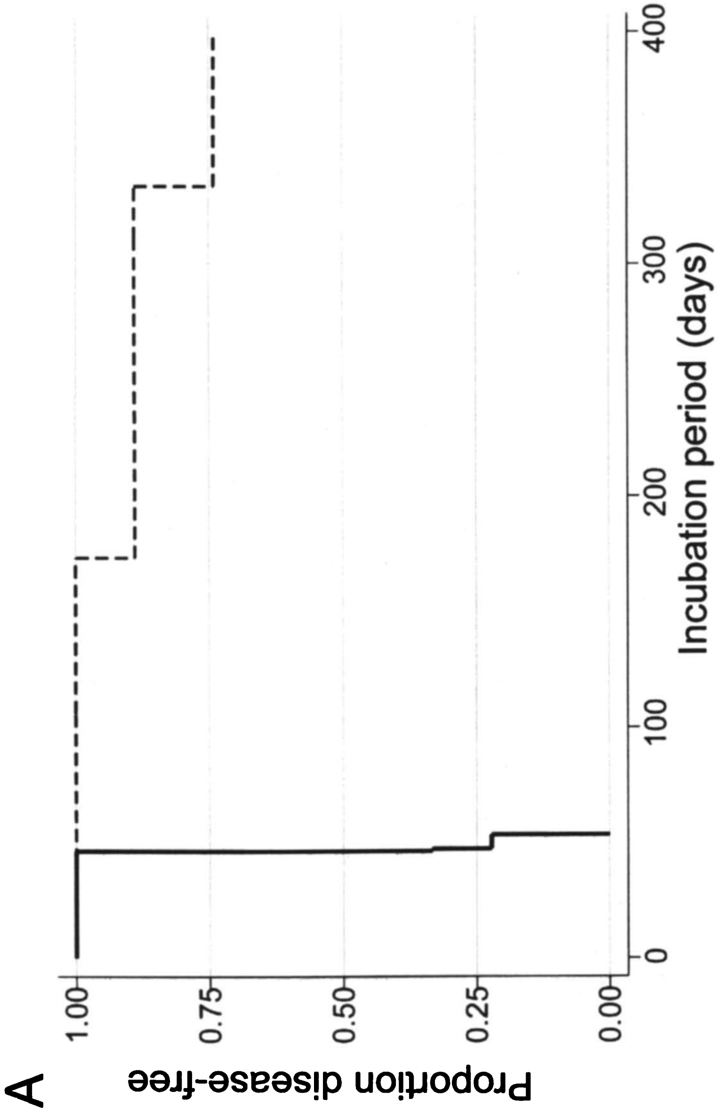

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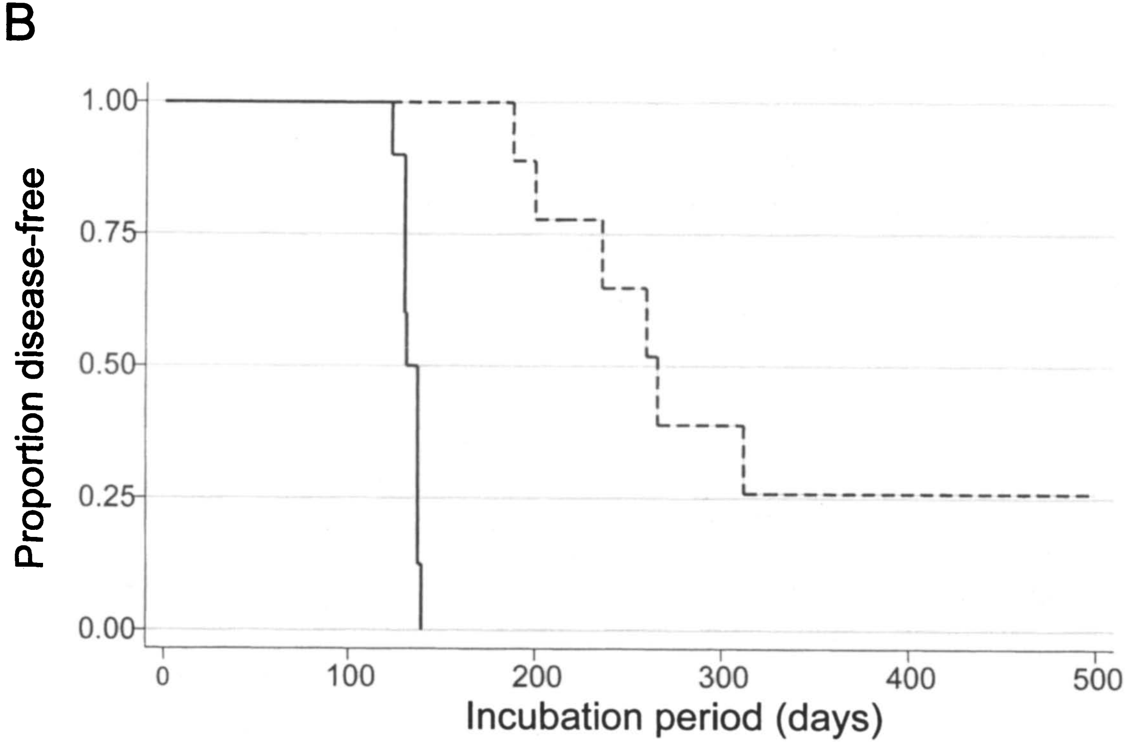

Figure 3. Kaplan-Meier survival curves for prion infected brain homogenate before (solid line) and after (dashed line) inactivation with acidic SDS (2% SDS, 1% AcOH) treatment for 30 min at 65 °C. (A) Sc237 prions bioassayed in Tg7 mice. (B) sCJD prions bioassayed in Tg22372 mice.

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Table 2. Inactivation of prions by 2% SDS + 1% AcOH at 121°C

	Hamster Sc237 prions in Tg7 mice				*Human sCJD prions in Tg23372 mice*			
	Homogenate		Wire		Homogenate		Wire	
	IP	Prop.	IP	Prop.	IP	Prop.	IP	Prop.
Positive control	46 ± 0.2	0.00	62 ± 0.3	0.00	146 ± 0.4	0.00	207 ± 1.1	0.00
Untreated, 15 min	344 ± 20	0.27	160 ± 7.3	0.00	221 ± 1.0	0.00	> 500	0.78
Untreated, 30 min	> 400	0.88	> 400	0.80	> 500	1.00	> 500	1.00
Untreated, 2 h	> 400	0.88	> 400	1.00	> 500	1.00	414 ± 15	0.27
2% SDS 1% AcOH, 15 min	> 400	1.00	> 400	1.00	> 500	1.00	> 500	1.00
2% SDS 1% AcOH, 30 min	> 400	1.00	> 400	1.00	> 500	0.88	> 500	1.00
2% SDS 1% AcOH, 2 h	> 400	1.00	> 400	1.00	> 500	1.00	> 500	1.00

Median incubation period (IP) ± standard error, in days, and proportion of animals remaining disease-free (Prop.) were calculated using Kaplan-Meier analysis.

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of PrP^{Sc} [47, 59, 60] and display distinct conformational stabilities as reflected in their resistance to inactivation by chaotropes and heat [47, 49, 61, 62]. These and other findings argue that the conformation of PrP^{Sc}, which reflects both the strain and the PrP sequence, determines the stability of the prion. Conformational stability profiles, as measured by sensitivity to denaturation by GdnHCl gave half-maximal ($Gdn_{1/2}$) values of 1.5 M and 1.8 M for Sc237 prions [46, 47], compared to a $Gdn_{1/2}$ value of 1.8 M for sCJD(MM1) prions [63]. However, a greater than 100,000-fold difference was demonstrated in the susceptibility of these strains to inactivation by acidic SDS [39]. It therefore not possible to extrapolate findings based on biophysical measurements to inactivation studies.

Both the human vCJD strain and the mouse 301V strain [64] are considered to represent the same strain as BSE, from which they were derived [49, 65]. However, they differ in their primary sequence: BSE is a bovine protein, vCJD is a human protein and 301V is a mouse protein, and they would not be expected to have the same inactivation characteristics. It is therefore essential that prion disinfection procedures are validated against the prion strain and species they are aimed to protect against.

Mechanism of acidic SDS inactivation

Exposure to acidic SDS appears to denature PrP^{Sc}: while SDS between pH 4.5 and pH 10.0 at room temperature is a poor denaturant, it becomes an excellent denaturant at pH ≤4.5 and ≥10 (**Figure 1**). In our initial experiments, denaturation of PrP^{Sc} was measured by the loss of PK-resistant PrP and a decrement in prion infectivity. Immunodetection of PK-resistant PrP using Western blotting has a dynamic range of ~100-fold while bioassays measure prions over a ~10^9-fold range as described above. In determining the mechanism of acidic SDS–mediated denaturation of PrP^{Sc}, immunoassays are useful but only prolonged bioassays are adequate to assess whether or not complete inactivation of prion infectivity has been achieved.

Prion inactivation with acidic SDS

From the foregoing studies, acidic SDS provides for the first time a means of completely inactivating human prions under relatively gentle conditions. sCJD(MM1) prions on steel wires were not completely inactivated by autoclaving at 121°C in the absence of acidic SDS (**Table 2**); this contrasts with Sc237 prions that were completely inactivated by extended autoclaving alone (**Table 2**). It is noteworthy that the prion-coated wires were not subjected to any procedures that might reduce the level of prions, such as washing, shaking, scrubbing, or sonicating. Such cleaning procedures are known to substantially reduce the titers of many different pathogens [17].

The data presented here document the efficacy of acidic SDS combined with autoclaving for complete inactivation of human and hamster prions in brain homogenates and on the surface of steel wires (**Table 2**). Acidic SDS combined with autoclaving should supplant routine autoclaving used to sterilize medical and dental equipment. For equipment such as fiber optic instruments that cannot be autoclaved, submerging such equipment in acidic SDS at 65°C will substantially reduce prion infectivity but not completely eliminate it (**Table 1**).

The results of inactivation studies described here argue that acidic SDS combined with autoclaving be applied immediately for sterilization of surgical and dental instruments. Because SDS is both a detergent and a protein denaturant, it should prove especially apt for sterilizing instruments with complex shapes, serrations, locks, bores, and crevices.

In addition to inactivating prions on the surfaces of surgical and dental instruments and diagnostic equipment, acidic SDS may prove useful in the sterilization of ophthalmologic instruments as well as the cleaning of operating theaters and diagnostic suites. Acidic SDS is likely to find use in the cleansing and sterilization of equipment used in the production of biopharmaceuticals. Additionally, acidic SDS might be applied in the cleaning of abattoirs, meat-processing plants, butcher shops, kitchens, and wherever mammalian products are prepared for human consumption. Acidic SDS might also be used on equipment employed in the rendering of offal. Because of its noncorrosive nature and ability to denature prions rapidly at relatively low temperatures, acidic SDS may be suitable for skin and wound cleansing.

In summary, it is important to recognize that procedures routinely used in medical and dental settings do not inactivate prions. That being the case, it may be prudent to institute acidic SDS protocols as configured in the studies presented here. Acidic SDS combined with autoclaving completely inactivated prion infectivity, even on steel surfaces. Inactivation of human and hamster prions mediated by acidic SDS occurs rapidly and can be achieved without boiling or autoclaving. Acidic SDS is noncorrosive and offers other practical advantages that make it suitable for widespread use.

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Chapter 4

Strategies for Inactivating Prions

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The purpose of this chapter is to briefly review general guidelines that are given to hospital central sterilization service departments for reprocessing instruments and medical devices that have been exposed to patients known or suspected to have Creutzfeldt-Jakob disease (CJD). CJD is a degenerative neurological disorder of humans with an incidence in the United States of approximately 1 case/million population/year. The disease is caused by a proteinaceous infectious agent that is referred to as a prion. The agent has no DNA or RNA. Prion diseases do not elicit an immune response, result in a noninflammatory pathologic process confined to the central nervous system, have an incubation period of years, and usually are fatal within one year of diagnosis. Disinfection and sterilization recommendations for CJD are based on the belief that infection control measures should be predicated on epidemiologic evidence linking specific body tissues or fluids to transmission of CJD, quantitative infectivity assays demonstrating that body tissues or fluids are contaminated with infectious prions, cleaning data using biological indicators and proteins, inactivation data on prions, the risk of disease transmission with the use of instruments or devices, and a review of other recommendations.

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The purpose of this chapter is to describe scientific rationale for the general recommendations used for the decontamination of instruments or medical devices that have been exposed to patients with Creutzfeldt-Jakob disease (CJD). CJD and a number of other fatal degenerative diseases of the central nervous system in humans or animals are caused by prions. This chapter is meant to be a very brief review of the subject. Recommendations for the sterilization of instruments and medical devices exposed to patients with prion disease (CJD) are based on studies that show prions are resistant to heat and chemical germicides. However, these studies use prion challenges that are enormous and unrealistic because they are in the form of slurries and bits of tissue. Because the principles of instrument cleaning are not taken into consideration as well as the invariably fatal outcome of CJD, most recommendations historically have been extraordinarily conservative.

Introduction

CJD is a degenerative neurological disorder of humans with an incidence in the United States of approximately one case per million population year (1). The disease is caused by a proteinaceious infectious agent or prion. CJD is related to other human transmissible spongiform encephalopathies (TSEs) that include kuru (0 incidence, now eradicated), Gertsmann-Straussler-Sheinker (GSS) syndrome (1 case per billion), and fatal insomnia syndrome (FFI) (less than 1 case per billion). Prion diseases do not elicit an immune response, result in a noninflammatory pathologic process confined to the central nervous system, have an incubation period of years, and usually are fatal within one year of diagnosis.

A recently recognized new variant form of CJD (vCJD) is acquired from cattle with bovine spongiform encephalopathy (BSE) or "mad-cow" disease. As of April 2005, a total of 165 vCJD cases had been reported worldwide: 155 in the United Kingdom (UK), six in France, and one each in Italy, Ireland, Hong Kong, and the United States. Each of the latter four patients had resided in the United Kingdom during the UK outbreak of BSE. Compared with CJD patients, vCJD patients are younger (29 vs. 65 years of age), have a longer duration of illness (14 vs. 4.5 months), and present with sensory and psychiatric symptoms that are uncommon with CJD. (2)

Prion caused diseases collectively have been referred to as "slow viral infections" (e.g., CJD or kuru in humans or scrapie in sheep or goats) and were thought to be caused by agents commonly referred to as "unconventional viruses". However, CJD and several other transmissible spongiform encephalopathies (TSEs) currently are believed to be caused by prions (3,4). These are small, proteinaceous unconventional agents referred to in the scientific literature as "PrP^{sc}." Proteins resist inactivation by procedures known to modify nucleic acids. Currently, there is no known immunologic response to the CJD agent. Traditional virologic methods have not been successful as tools to characterize the etiologic agent. PrP^{sc} is an isoform of a normal protein (PrP^{c}) concentrated in brain tissue. PrP^{sc} and PrP^{c} are identical chemically but have different molecular shapes. The normal protein in uninfected individuals is sensitive to the action of proteinase K, whereas the CJD protein, PrP^{sc} is resistant to this enzyme. As mentioned the two isoforms, while identical in amino acid sequence, differ in their three-dimensional conformation and glycosylation patterns. (Figure 1) PrP^{c} is converted to PrP^{res} in a process as yet undetermined, but the conversion is thought to involve posttranslation modification of the protein (5)

The agents of CJD and other TSEs exhibit an unusual resistance to conventional chemical and physical decontamination methods. Since the CJD agent is not readily inactivated by conventional disinfection and sterilization procedures and because of the invariably fatal outcome of CJD, the procedures for disinfection and sterilization of the CJD prion have been both cautious and controversial for many years.

CJD occurs as both a sporadic and familial disease. Less than 1% of CJD episodes have resulted from health-care-associated transmission and only two confirmed cases and four unconfirmed cases have been associated with reprocessed surgical instruments; these cases occurred over 25 years ago in Europe. No cases of CJD or vCJD associated with surgical or medical instruments have been reported since that time.

The vast majority of health-care-associated transmission cases have resulted from use of contaminated tissues or grafts. Iatrogenic CJD has been described in humans in three circumstances:

a) after patients received extracted pituitary hormones (more than 130 cases);
b) after patients received an implant of contaminated grafts from humans (cornea, three cases; dura mater, more than 110 cases);
c) and, as mentioned above, after use of contaminated medical equipment and surgical instruments on patients undergoing invasive procedures (two confirmed and four unconfirmed cases) (5,6).

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Iatrogenic CJD Transmission

All known instances of iatrogenic CJD transmission have resulted from exposure to infectious brain, pituitary, or eye tissue. Tissue infectivity studies in experimental animals have determined the infectiousness of different body tissues (7). Tissues that have the highest prion concentration are brain and dura mater. Transmissibility is directly related to the concentration of prions in tissues.

Transmission via stereotactic electrodes is the only convincing example of transmission via a medical device (8). The electrodes had been implanted in a

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Prion = Proteinaceous Infectious Particle

PrP^c = Prion Protein cellular =normal or "healthy" prions

PrP^{sc} = rogue prions/ pathologic isoform

PrP^c and PrP^{sc} are the same chemical but have different shapes

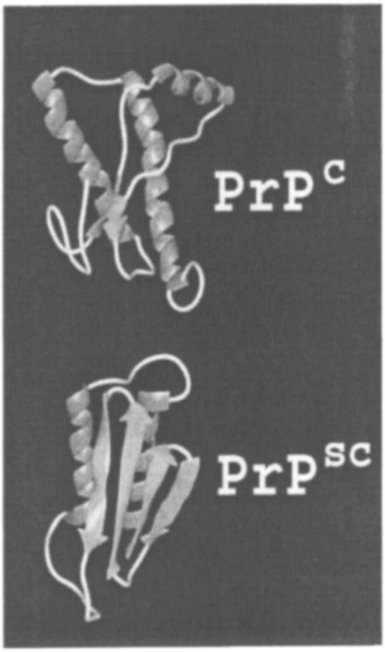

Figure 1. Images of Prions. (See page 1 of color inserts.)

patient with known CJD. The electrodes were cleaned with benzene and then "sterilized" by soaking them in 70% alcohol and exposing them, during storage at room temperature, to formaldehyde vapor produced by a formaldehyde generator using solid paraformaldehyde. Two years later, these electrodes were retrieved and implanted into a chimpanzee in which the disease developed. This method used to "sterilize" these electrodes would not currently be considered an adequate method for sterilizing medical devices.

Retrospective studies suggest that four other episodes may have resulted from use of contaminated instruments in neurosurgical operations (9). An index CJD case was identified in only one case; and in this instance, the surgical instruments were cleaned with soap and water and then exposed to dry heat for an unspecified time and temperature. The other three cases had no associated index case; these patients did have a neurosurgical procedure within a two-year period before being diagnosed with CJD.

All six cases of CJD associated with neurosurgical instruments occurred in Europe between 1953 and 1976, and details of the reprocessing methods for the instruments are incomplete. There are no known episodes of CJD attributable to the reuse of devices contaminated with blood or to transfusion of blood products.

The infrequent transmission of CJD via contaminated medical devices probably reflects the inefficiency of transmission except for neural tissue and the effectiveness of conventional cleaning and current disinfection and sterilization procedures (9).

To minimize the possibility of use of neurosurgical instruments that have been potentially contaminated during procedures performed on patients in whom CJD is later diagnosed, health care facilities should consider using the sterilization guidelines outlined below for neurosurgical instruments used during brain biopsy done on patients in whom a specific lesion has not been demonstrated (e.g., by magnetic resonance imaging or computerized tomography scans). Alternatively, neurosurgical instruments used in such patients could be disposable or the instruments could be quarantined until the pathology of the brain biopsy is reviewed and CJD excluded (9).

Prion proteins are concentrated in the tissues of the central nervous system (CNS); these are considered the "high-risk" tissues for CJD (Table I). Tissues identified as "medium-risk" are cerebrospinal fluid (CSF), lymph node, spleen, pituitary gland, and tonsil. "Low-risk" tissues include bone marrow, liver, lung, thymus, and kidney. Tissues and body substances that carry little or no risk of transmitting CJD include blood, feces, urine, skin, peripheral nerves, saliva, gingiva, and other organ systems

The transmissibility of the CJD agent has been demonstrated in that disease can be induced in laboratory animals by percutaneous inoculation of infective material (i.e., brain tissue or CSF), but not by simple direct contact. Transmission of CJD has not been associated with environmental contamination or fomites. There are several categories of CJD cases classified on the basis of

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TABLE I. Risk of CJD Transmission as a Function of Type f Tissue and Expected Relative Concentration of CJD Agent

RISK	TISSUE
High - More than 50% of CJD infectivity tests positive	Brain, dura mater, cornea
Medium - 4-50% of CJD infectivity tests positive	Cerebrospinal fluid, kidney, liver, lymph node, spleen
Low to None - Infectivity tests negative	Blood, urine, adrenal gland, feces, heart, bone marrow, muscle, nasal mucus, peripheral nerves, saliva, gingiva, sputum, tears

(Adapted from Geertsma and van Asten (19); Asher (5) and Brown (7) transmission and/or pattern of occurrence. These are Sporadic, Familial, Iatrogenic, Occupational, and the New Variant form of CJD.

Sporadic cases of CJD account for approximately 90% of the disease in North America. The exact mode of acquisition or transmission of sporadic CJD in humans is not known. Person-to-person transmission via skin contact has not been documented.

Less than 10% of CJD episodes may be related to a genetic predisposition. Genetic studies have revealed autosomal dominance for a gene on chromosome 20 to be an important factor in these cases. Host genetics may also play a role in determining susceptibility or resistance to the development of the sporadic disease, but as yet this is undefined.

Iatrogenic episodes of CJD have been recognized (Table II), and transmission has occurred via percutaneous exposure to, or brain contact with, medical instruments contaminated with prion/tissue residues or transplantation of CNS brain tissue as described. Iatrogenic spread from person-to-person is exceedingly rare, but it has occurred after transplantation of corneas or dura mater grafts obtained from infected individuals, and the use of contaminated neurosurgical instruments or stereotactic depth electrodes previously used on infected individuals. In the latter instance, brain tissue remained on the instruments due to insufficient or inappropriate cleaning, which precluded effective treatment (i.e., sterilization) of the instrument for re-use. (10)

Although isolated episodes of CJD have occurred in approximately 24 physicians and other health care workers (including two neurosurgeons, one pathologist, nine nurses, and two histology technicians), epidemiologic studies have documented occupationally acquired CJD in only three of these workers. All three had percutaneous exposures to high-risk tissues in laboratory settings. Despite the natural concern these reports produce among some health professionals, it is important to recognize that the incidence of CJD in these groups does not exceed what would be expected by chance alone. There have been no documented reports of transmission of disease from patients to hospital or mortuary staff. Epidemiologic analysis of health care workers, farmers, butchers, or spouses of patients revealed no overall increased risk of acquiring CJD due to occupational factors.

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Prion Inactivation Studies

Historically a number of investigators have studied the inactivation of prions by disinfection and sterilization processes. However most of these studies do not reflect the reprocessing procedures in a clinical setting:

a) These studies have not incorporated a cleaning procedure that normally reduces microbial contamination by 4 log10 and reduces protein contamination (9).

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TABLE II. Reported Mechanisms of Iatrogenic or Occupationally Acquired Episodes of CJD

Source of CJD Agent	NUMBER OF CASES*
Depth Electrodes	2**
Neurosurgery	4***
Corneal Transplantation	3
Dura Mater Transplantation	169
Pituitary Hormone and Gonadotrophin	179
Total	**348**

* As of May 2005
** Confirmed cases associated with instruments
*** Unconfirmed cases associated with instruments

b) Most prion inactivation studies have been done with tissue homogenates dried onto carriers. The protective effect of the tissue and the drying of the tissue may explain, in part, why the CJD agent is difficult to inactivate in these experimental studies.

c) Results of inactivation studies of prions have been inconsistent due to the use of differing methodologies, which have varied by prion strain, prion concentration, test tissue (intact brain tissue, brain homogenates, partially purified preparations), test animals, duration of follow-up of inoculated animals, exposure container, method of calculating log-reductions in infectivity, concentration of the disinfectant at the beginning and end of an experiment, cycle parameters of the sterilizer, and exposure conditions.

Despite these limitations, there is some consistency in the results (9). In order to provide scientifically based recommendations, research should be undertaken in which actual medical instruments are contaminated with prions (including variant CJD), cleaned, and then subjected to either conventional sterilization or disinfection or special prion reprocessing.

The disinfection studies mentioned above showed that many, but not all, disinfection processes fail to inactivate clinically significant numbers of prions. There are four chemicals that reduce the prion titer by more than 3 log10 in 1 hour: chlorine (sodium hypochlorite), a phenolic (based on ortho-phenylphenol, p-tertiary-amylphenol, and ortho-benzyl-para-chlorophenol) at concentrations greater than 0.9%, guanidine thiocyanante, and sodium hydroxide. Of these four chemical compounds, chlorine has provided the most consistent prion inactivation results. However, the corrosive nature of chlorine makes it unsuitable for many devices, such as surgical instruments and endoscopes.

Prions also exhibit an unusual resistance to conventional physical decontamination methods (9). While there is some disagreement on the ideal time and temperature cycle for steam sterilization, the recommendation for 134C for greater than or equal to 18 minutes (prevacuum) and 132°C for 60 minutes (gravity-displacement) are based on the scientific literature (9) and have been shown to provide significant but not complete reduction of infectivity under worst-case conditions (5).

Recent Studies

Within the last two years there have been a number of studies that show a number of processes and germicides besides high moist heat and exposure to strong hypochlorite or sodium hydroxide solutions can effectively inactivate prion challenges. The basic approach has been to use experimental animals such as mice or hamsters. Steel wires inoculated with a specific prion challenge are surgically implanted in animals, intracerebrally, and the animals are followed.

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Yan, et al (11) using hamsters showed that a hydrogen peroxide gas sterilizer had a limited effect, but when an alkaline detergent was used in combination with a hydrogen peroxide gas sterilization cycle (4 injections) complete inactivation of the prion challenge was achieved. Five to 6 logs of prion reduction was achieved using an alkaline detergent and a standard hydrogen peroxide gas sterilization cycle (2 injections); or the alkaline detergent treatment alone.

Fichet, et al (12) using hamsters showed that standard chemical decontamination methods (1N NaOH, NaOCl 20 000 ppm) and autoclaving in water at 134°C reduced infectivity by >5·6 $\log^{10}$ lethal doses; autoclaving without immersion was somewhat less effective (4–4·5 log reduction). Three milder treatments, including a phenolic disinfectant, an alkaline cleaner, and the combination of an enzymatic cleaner and vaporised hydrogen peroxide (VHP) were also effective.

Race and Raymond (13) used hamsters as an animal bioassay, and found that a commercially available environmental phenolic disinfectant, Environ LpH, significantly reduced a prion challenge.

Baier, et al (14) using hamsters showed that a routinely available alkaline cleaner could reduce the infectivity of a prion challenge significantly. Lemmer et al (15) showed under appropriate conditions, relatively mild reagents such as 0.2 % sodium dodecyl sulfate (SDS) and 0.3% NaOH (pH 12.8), a commercially available alkaline cleaner (pH 11.9–12.2), a disinfectant containing 0.2 % peracetic acid and low concentrations of NaOH (pH 8.9) or 5 % SDS (pH 7.1) exerted potent decontaminating activities on PrP^{Sc}/PrP27-30 attached to steel surfaces

Jackson et al (16) showed that a combination of proteinase K and Pronase, in conjunction with SDS, degraded PrP(Sc) material from highly concentrated vCJD-infected brain preparations to a level below detection and no infectivity could be demonstrated.

Baxter et al (17) investigated radio-frequency (RF) gas-plasma treatment as a method for removing both the protein debris and prion infectivity. Stainless-steel spheres contaminated with scrapie prions and a variety of used surgical instruments, which had been cleaned by a hospital sterile-services department, were examined both before and after treatment by RF gas plasma, using scanning electron microscopy and energy-dispersive X-ray spectroscopic analysis. Transmission of scrapie from the contaminated spheres was examined in hamsters by the peripheral route of infection. RF gas-plasma treatment effectively removed residual organic residues on reprocessed surgical instruments and gross contamination both from orthopedic blades and from the experimentally contaminated spheres. The infectivity of prions adsorbed onto metal spheres could be removed effectively by gas-plasma cleaning with argon/oxygen mixtures as well as the cleaning method used.

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Petetz et al (18) (Kurt Giles, presented their data at our Symposium *BIOCIDES OLD AND NEW: WHERE CHEMISTRY AND MICROBIOLOGY MEET* -PacifiChem 2005. His paper is in this book) tested various concentrations of SDS and acetic acid (AcOH) and found that the duration and temperature of exposure acted synergistically to inactivate both hamster Sc237 prions and human sporadic Creutzfeldt-Jakob disease (sCJD) prions. The inactivation of prions in brain homogenates and those bound to stainless steel wires was evaluated by using bioassays in transgenic mice. sCJD prions were significantly more resistant to inactivation than hamster derived Sc237 prions, demonstrating that inactivation procedures validated on rodent prions may not be able to be extrapolated to inactivation of human prions. Using acidic SDS combined with autoclaving for 15 min., human sCJD prions bound to stainless steel wires were eliminated.

G. McDonnell, a participant in our Symposium, made a number of pertinent comments during the discussion period. He pointed out that as is known from bacterial and viral studies, the test method can be an important factor in determining degree of resistance and the sterilization/disinfectant method will also show differences. He indicated that this is also seen with prions. If one considers the test method alone (original brain homogenate: lipid content, preparation method; surface inoculation; animal responsiveness etc) there could be a significant source of test-to-test, and laboratory-to-laboratory variability. Some studies have shown greater resistance of BSE/vCJD strains with enzymatic and disinfectant formulations, while others have shown that scrapie strains are much more resistant.

Prevention of CJD Transmission by Surgical Instruments

Historically, recommendations for inactivating the agent of CJD have been based on studies using infected tissues and injecting animals known to be susceptible to CJD. Many of the existing recommendations are based on the assumptions that exposure to any tissue, body fluid, secretion, or excretion from a CJD patient will result in a transmissible infectious dose of CJD, and that no conventional processing regimen of cleaning followed by disinfection or sterilization will be effective in rendering the device or fomites safe for reuse. However, based on the epidemiology of iatrogenic and health-care-associated (nosocomial) episodes of CJD mentioned above, it is clear that the only exposures in patient-care settings that have resulted in infection are those instances involving devices that cannot be cleaned and that are contaminated with high-risk tissue from the central nervous system.

There have been other approaches that consider tissues containing the highest prion load to carry the highest risk of transmission by instruments (19,20,21,9)

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Rutala and Weber (9) point out that the disinfection and sterilization recommendations for CJD in should be based on epidemiologic evidence linking specific body tissues or fluids to transmission of CJD, quantitative infectivity assays demonstrating that body tissues or fluids are contaminated with infectious prions, cleaning data using BIs and proteins, inactivation data on prions, the risk of disease transmission with the use of the instrument or device, and a review of other recommendations

The three parameters they integrated into strategies for disinfection and sterilization processing are as follows:

a) Risk of the patient for having a prion disease: High-risk patients include those with known prion disease; those with rapidly progressive dementia consistent with possible prion disease; those with a familial history of CJD, GSS, or FFI; patients known to carry a mutation in the PrP gene involved in familial TSEs; patients with a history of dura mater transplants; and patients with a known history of cadaver-derived pituitary hormone injection.

b) Comparative infectivity of different body tissues (e.g., the prion load): High-risk tissues include brain, spinal cord, and eye. All other tissues are considered low or no risk.

c) Intended use of the medical device: Critical devices are defined as devices that enter sterile tissue or the vascular system (e.g., implants, curettes). Semicritical devices are defined as devices that contact nonintact skin or mucous membranes (e.g., endoscopes).

Devices Contaminated with High-Risk Tissues (summarized from Rutala and Weber-9)

Those devices that are impossible or difficult to clean should be discarded, or decontaminated initially by autoclaving at 132-134°C for 18 minutes in a prevacuum sterilizer, or 121°C for one hour in a gravity displacement sterilizer, or soaked in 1 N NaOH for one hour before terminal cleaning, wrapping, and sterilization by conventional means.

Those devices that are constructed such that cleaning procedures result in effective tissue removal can be cleaned and then sterilized by autoclaving at 132-134°C for 18 minutes in a prevacuum sterilizer, or 121°C for one hour in a gravity displacement sterilizer.

Devices Contaminated with Medium- or Low-Risk Tissues

These devices can be cleaned and disinfected or sterilized using conventional protocols of heat or chemical sterilization, or high-level disinfection.

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Among the most frequently asked questions are those regarding appropriate reprocessing protocols for flexible endoscopes after use on a patient with CJD. The current guidelines for cleaning and disinfection of these instruments need not be changed. To further minimize the risk of transmission of infection in general for any disease when an invasive procedure (i.e., biopsy) is done during endoscopy, several options may be considered. If the device accessory (e.g., biopsy forceps) is difficult to clean, the accessory may be discarded as a single-use item. If the accessory is heat-stable, it should be cleaned thoroughly with an ultrasonic cleaner and reprocessed by steam autoclaving.

Environmental (Housekeeping) Surfaces

Environmental surfaces would not be expected to be associated with transmission of CJD to health care workers or patients. Floors, walls, counter tops, or other housekeeping surfaces in medical wards, autopsy rooms, and laboratories that are contaminated with high-risk tissues should cleaned with a suitable detergent in the conventional fashion. A 1:10 dilution of chlorine bleach can be used to spot decontaminate visible residues of tissue before cleaning.

Summary

Prion diseases are rare and do not constitute a major infection control risk. Nevertheless, reprocessing strategies for surgical instruments exposed to high-risk tissue of patients known or suspected of having CJD or vCJD represent an exception to conventional disinfection and sterilization practices. Current strategies for CJD disinfection and sterilization are based on consideration of epidemiological data, infectivity data, and cleaning and inactivation studies. Guidelines for management of CJD infected patients and patient equipment should be modified as scientific information becomes available. There are a number of research groups currently that are performing studies on prion inactivation and the results have shown that prion inactivation can be accomplished without the use of corrosive solutions and high temperatures.

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Chapter 5

Biofilms and Biocides: Growing Consistent Monoculture Biofilms for Biocide Assessment

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The assessment of biocide efficacy for the control of biofilm growth from flowing water, depends on the quality of the biofilm. In turn the biofilm quality depends on the conditions and their interaction, under which the biofilm was grown. It is vital therefore, in order to make valid and reliable comparisons of biocide activity, that biofilms are established and grown under carefully controlled conditions. Data demonstrate the effect of variables on the extent and quality of biofilms and on the effectiveness of biocides. A form of laboratory apparatus that has been found suitable for growing biofilms for biocide assessment is discussed.

Introduction

Biofilms can develop in a number of industrial operations, including food processing, machining operations where the circulation of liquids is involved but by far, the largest area is in cooling water systems. Many industrial processes involve the cooling of product or intermediate streams, liquid or gas or mixtures. The cooling medium is usually, though not exclusively, water. On account of cost, convenient natural sources of water are utilised, including lakes, rivers and canals. If the industry is located close to a coastline, seawater may be employed.

In New Biocides Development; Zhu, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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The water from these sources usually contains decomposing natural material such as plants, leaves, dead insects and animal carcasses, in addition to dissolved oxygen, and inorganic salts as well as living organisms. The living matter may include macro and micro-organisms. The former can include mussels, barnacles and serpulid worms and the latter often includes algae, fungi and bacteria. In cooling systems both macro and micro-organisms can cause serious loss of efficiency with consequent financial and other penalties.

As an example, the efficiency of modern power stations is very dependent on effective steam condensers for enhancing the utilisation of the high pressure steam to drive the turbo-generators. As many power station condensers are cooled by the use of naturally occurring water, the heat transfer surfaces often become fouled with crystallised salts, particulate material, and living micro-organisms. Macro-organisms present in the source water are generally removed by some form of filtering before the water is pumped through the system. Macro-organisms that remain in the water can represent a nuisance in relation to effective plant operation in some situations.

Usually the steam condensers are tubular, the steam condensing on the outside of tubes through which the cooling water is passing. Unless effective precautions are taken, unwanted deposits will accumulate on the inside of the tubes. These deposits act as an insulating layer thereby restricting the heat transfer and reducing efficiency. Where untreated water from natural sources is used for cooling purposes, biofilms are likely to occur. In general, it is bacteria that thrive under the prevailing conditions, since the water temperature is close to the optimum for growth, the flowing water contains nutrients and the mass transfer conditions are conducive to the delivery of colonising bacteria and nutrients. Furthermore the removal of waste products can also be facilitated by enhanced mass transfer. The presence of algae and fungi is restricted since the former require light to facilitate metabolism and fungi need a consumable substrate (the condenser tubes are of course metal). Suitable conditions therefore, do not exist for these organisms to survive on the water-side of the condensers. If the system is recirculating and there is a cooling tower to remove the heat collected by the water in the condensers, it is possible that fungi could grow on suitable packing in the tower. Modern plastic tower packing material obviates the problem to a large extent. Algae could flourish in the water collected in the basin under the tower, unless suitable control measures are in place.

Restriction of heat transfer is not the only detrimental effect of biofilms on the condenser tubes; the biofilm also reduces the water flow area. For a given volumetric flow rate therefore, the water velocity is increased. Higher velocity increases the energy required to pump the water through the system. Furthermore the surface of the biofilm in contact with the flowing water is "rough" offering a further restriction on the water flow and an attendant increase in the energy required for pumping.

The common method of restricting the development of these detrimental biofilms, is to dose the water with a suitable biocide. The preferred biocide for many years has been chlorine because of its effectiveness, relatively low cost and ready availability. It is however not environmentally friendly since it reacts with the organic matter in the cooling water to produce chloro-compounds that are considered to be carcinogenic. Because the water is returned to the source and often the water in rivers is abstracted downstream for the production of town's water, these chloromethanes as they are collectively known, have to be removed to meet local water quality regulations. As can be appreciated, the removal costs are quite high. As a result there is a continual search for so called environmentally friendly biocides, that are effective at reasonable cost, short-lived and break down to environmentally acceptable by-products.

In order to develop these effective biocides for industrial application it is necessary to carry out carefully controlled tests in the laboratory, prior to on-site testing. If these preliminary tests are not undertaken there could be serious problems if attempts are made to introduce new biocides directly at the full-scale level. Experimentation on a large scale is on the whole, unacceptable. It is important that the preliminary testing is carried out in a robust scientific manner so that comparisons and assessments can be made with confidence.

As might be expected, the "quality" of a biofilm very much depends on the conditions under which it was established and developed. In addition, as it consists of living matter, the biofilm responds to any changes in the local conditions that may occur with corresponding changes in structure and viability. Under certain conditions notably low water velocity and high nutrient levels, the biofilm has an open structure, with a tendency to slough. On the other hand, high water velocity and low nutrient levels, tend to give a robust compact biofilm that is more resistant to the prevailing shear forces than a more open structure. The quality and thickness of the biofilm will of course, have a profound influence on its resistance to biocide action.

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The Establishment of a Biofilm

A series of steps can be identified that lead to the colonisation and the development of a biofilm on a solid surface from water flowing across that surface. Characklis and Cooksey (1) have identified five such stages:

1. Transport of micro-organisms and organic macromolecules to the wetted surface.
2. Adsorption of the macro-organic material onto the wetted surface.
3. Attachment of micro-organisms to the conditioned surface.
4. Growth of the biofilm.
5. Detachment or sloughing of parts of the biofilm under the influence of the flow conditions.

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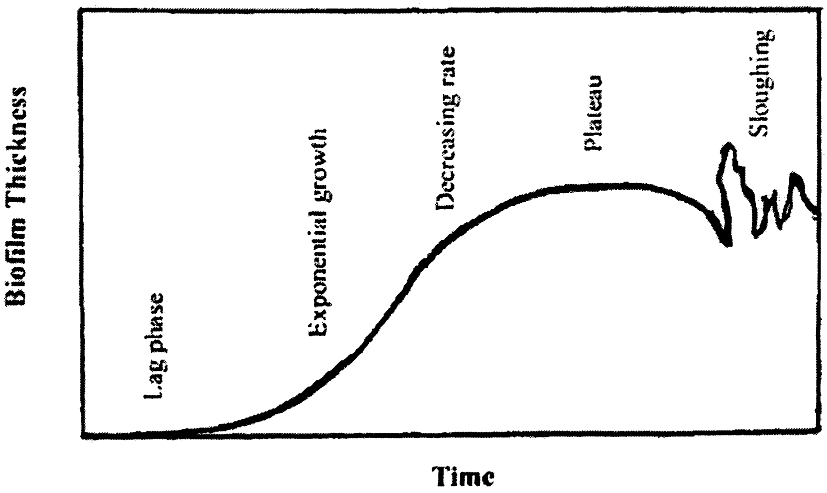

Figure 1. Biofilm development with time.

Biofilm Formation

The effectiveness of biocides is very much a function of their interaction with the conditions related to the development of the biofilm. An idealised graph of the development of a biofilm over a period of time, is presented in Fig. I. It is self explanatory but there are some aspects worthy of note:

1. The so called lag phase involves the colonisation of the surface by micro-organisms. As might be expected this can be a relatively slow process. Not only do the organisms have to reach the surface, but their subsequent attachment is thought to be dependent on the presence of the conditioning layer of organic molecules (2). Before colonisation can begin in a perceivable way, the virgin surface has to receive sufficient organic macromolecules to form a conditioning layer. The process depends on diffusion that can, under certain conditions, be a slow process.
2. Once the conditioning layer has been developed and bacteria are attached to the surface, rapid exponential growth can be initiated.
3. With the passage of time and the magnitude of the operating conditions, particularly the water velocity, the rate of growth begins to fall off, due in part, to the reduced availability of nutrients and the impact of removal forces acting on the biofilm.

4. It is possible to reach a plateau where the development of new biofilm is limited by the restrictions of nutrient availability and the loss of biofilm as a result of biofilm sloughing.

Factors that affect the transport of micro-organisms, particles, and soluble nutrients to and from the solid surface, will affect the development of biofilms and thereby control the ultimate shape of the biofilm accumulation versus time curve. The general shape of the growth curve for biofilms will be seen in much of the graphical experimental data presented later in this chapter.

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Background Physical Science

The deposition and subsequent growth of biofilms from flowing water moving across a surface, is very dependent on conditions within the water, particularly the fluid velocity. Two basic conditions are recognised; laminar flow (sometimes referred to as "streamline flow") and turbulent flow. At low velocity the flow is likely to be laminar and the mixing within the bulk fluid is essentially due to Brownian motion. On the other hand under turbulent conditions there is mixing due to the movement of the fluid within itself. Where a fluid in turbulent flow is moving across a surface, the frictional drag on the fluid imposed by the surface, means that the layers of fluid close to the surface are slow moving and in laminar flow, usually referred to as the “viscous sub-layer” Furthermore the fluid in actual contact with the solid surface is stationary. This whole region is generally referred to as the "boundary layer" and because the mixing here is due mainly to Brownian motion the transport of material in the flowing fluid to the surface is restricted. This applies to both solid particles and dissolved substances. The boundary layer can be likened, in its restriction to the movement of particles and dissolved substances, to an insulating layer that restricts the flow of heat. As the bulk velocity of the fluid is increased the turbulence increases and the thickness of the boundary layer is reduced. It has to be stated however, that in general no matter how high the bulk velocity there will always be a boundary layer. As velocity increases the shear forces at the solid surface rapidly increase in proportion to the square of the velocity. Any material on the wall, such as a biofilm, that is not strongly attached to the surface is likely therefore, to be removed.

The transport of material across the boundary layer will in general, also require a "driving force" i.e. a concentration gradient. This requirement will apply to dissolved substances and to most situations involving solid particulate material. Under some circumstances however, particles may acquire sufficient momentum to "coast" across the boundary layer to the surface. The frictional drag on the particle will reduce its velocity however, so that its momentum is removed and it may not reach the surface.

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The condition of the surface, particularly its roughness, will also influence the flow conditions and the ability of the surface to retain solid deposits, such as micro-organisms. The electro - chemical properties of the surface will also be a factor in the deposition and retention of solid material. These properties will be modified in the presence of deposits.

From this brief discussion of fluid flow it may be seen that the accumulation of biofilms, in terms of colonisation, the availability of nutrients, biofilm retention and indeed, the application of biocides, is very dependent on the flow conditions.

Velocity, Nutrient and Microbial Concentration, and Temperature Effects

Research into the relationship between bacterial concentration and water velocity has been reported.(3). Using *Pseudomonas fluorescens* at different cell concentrations (1, 2.2 and 3.6 x 10^6 cells/ml) in water that was passed through aluminium tubes at velocities of 0.6 and 1.8-1.9 m/s, the weight of the resulting biofilms was obtained. The results of the experiment are given in Figure 2. Low flow velocity coupled with low cell concentration, provides only a limited biofilm since the opportunity for cells to have sufficient momentum to cross the

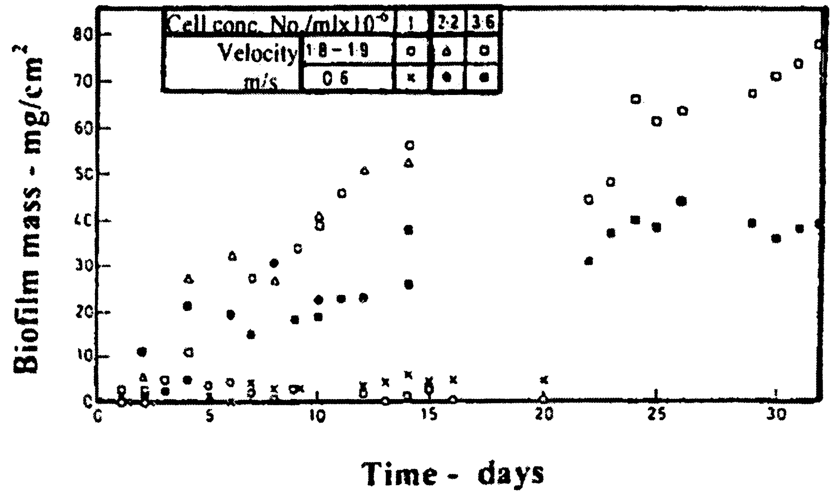

Figure 2. Biofilm weight per unit area with time under stated condition (3).

boundary layer to the surface is restricted. The maximum microbial accumulation occurred at the higher velocity of around 1.8 m/s and the highest cell concentration of 3.6 x 10^6 cells/ml producing a biofilm about twice the weight per unit area of the biofilm produced at 0.6m/s and with the same cell concentration in the flowing water. The higher cell concentration and water velocity enabled a relatively large number of cells to reach the surface in about 32 days.

Work by Patel (4) using a rectangular test section in which he grew biofilms without nutrients present, demonstrated the effect of velocity on the resulting biofilm. The thickness was determined using an electrical conductance technique. *Pseudomonas fluorescens* was again used as the micro-organism in these tests since the species *Pseudomonas* is a known slime former. Indeed it is the reason that it is used to obtain much of the data reported in this chapter. Fig.3 provides data on the colonisation of a surface. It will be seen that the thickness of the biofilm is very dependent on the velocity. With no nutrients present the development of the biofilm is dependent on the inertia of the individuals or clusters of micro-organisms to enable them to pass through the boundary layer and reach the solid surface. Depending on the prevailing conditions they may remain and become bonded to the surface via the conditioning layer, or they may be swept away by the effects of the flow conditions. It is apparent that the thickest biofilm occurs at the highest velocity studied i.e.2 m/s. This could be anticipated, since the highest momentum the micro-organisms can attain will be at the highest velocity and hence they will have the greatest opportunity to reach the surface. The shape of the curve closely resembles the idealised curve (Fig. I). It is to be noticed too, that the ultimate thickness of the biofilm is small and very much in contrast to the accumulation of biofilm when nutrients are present.

Figure 4 (5) illustrates clearly the effect of water velocity on the growth of biofilm under otherwise identical conditions, It can be seen that at the higher velocity of 1.27 m/s the thickness of the biofilm *(Pseudomonas fluorecsens),* is very much less than the biofilm grown from water flowing at 0.86 m/s.

Some recent work (6) measured the adhesive strength of biofilms grown at different velocities. Figure 5 shows that the adhesive strength of biofilms is very dependent on the water velocity under which they are grown. The adhesive strength increases as the velocity increases.

An interesting aspect of work by Bott and Miller (7) was the effect of the availability of bacteria in the flowing water, in contrast to the availability of nutrients. Data on Figure 6 demonstrates that once a surface has been colonised provided nutrients are available (in this work 4 mg/l in terms of glucose), the biofilm will be sustained and is capable of continued growth due to reproduction rather than to deposition of further cells.

On the other hand if the availability of nutrients is restricted there is likely to be an immediate effect on the biofilm. Figure 7 illustrates the point. The conditions are similar to those on which Figure 6 is based. At a velocity of 0.5

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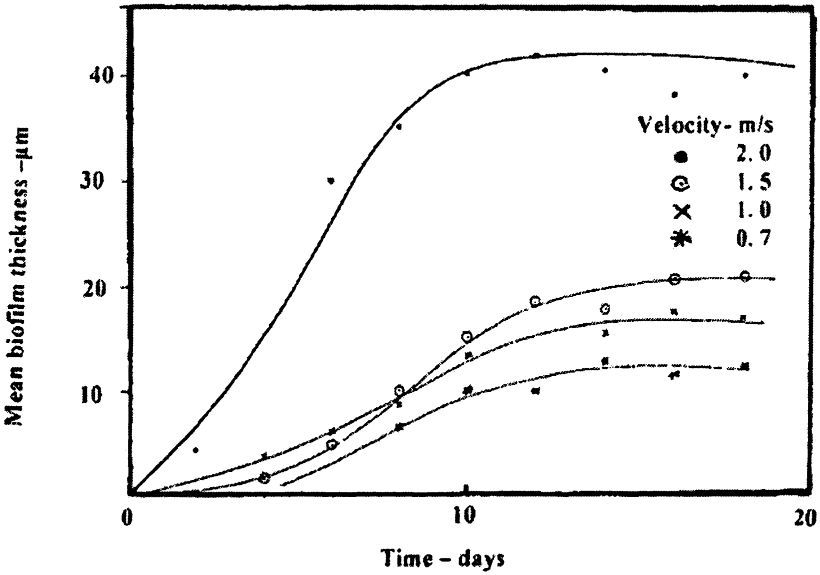

Figure 3. Biofilm thickness development with time at different water velocities with no nutrient present in the flowing water (4).

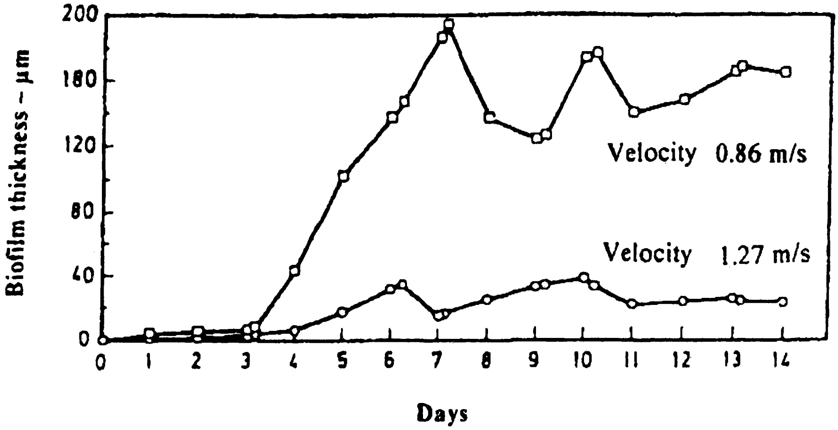

Figure 4. Biofilm thickness development with time for two different water velocities (5).

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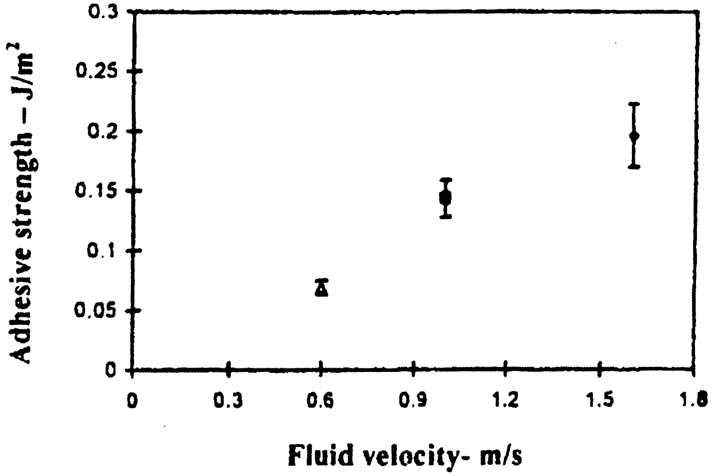

Figure 5. Biofilm adhesive strength at three water velocities. The error bars represent the standard error of the mean (5).

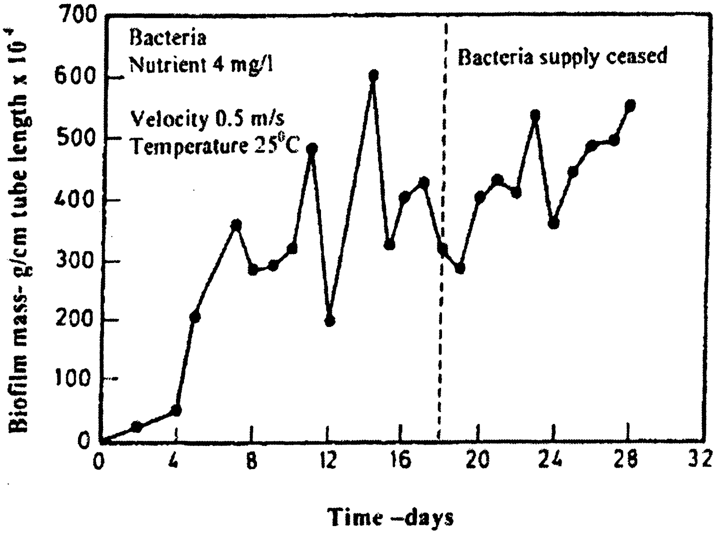

Figure 6. Biofilm growth with and without contaminating bacteria in the flowing water (7).

m/s when the nutrient supply is stopped there is an immediate fall in the amount of biofilm on the surface of the aluminium tube. It is apparent that the biofilm produced is sustainable when nutrients are available i.e. any removal of biofilm as a result of the relatively high shear forces produced by the high velocity, is replaced by growth.

The available nutrient at the base of the biofilm is also an important criterion for the viability of a biofilm and presumably as a result, its resistance to the effects of removal forces. The diffusion of oxygen through biofilms of the aerobic bacterium *Pseudomonas fluorescens* grown under different water velocities is given in Figure 8 (8). The oxygen profiles for the three water velocities namely 2.0, 1.0 and 0.7 m/s indicate that an increase in water velocity increases the oxygen availability in the lower layers of the biofilm, at a thickness of 100μm there is a higher level of oxygen than at the lower velocities of 1.0 and 0.7 m/s. However as the biofilm thickness approaches or exceeds 300μm, there is little difference between the oxygen availability at the three velocities. These results are in general agreement with the theory of mass transfer, which would suggest that there is greater mass transfer as velocity increases, since the thickness of the viscous sub-layer adjacent to the surface of the biofilm, is thinner at higher bulk velocities. However it has to be recognised that as oxygen diffuses or is carried through the biofilm interstices and channels by the ingress of water, it will be imbibed on the way by resident cells. It is more than likely also that the metabolism of the cells remote from the flowing water will be adversely affected by oxygen limitation that in turn may assist the removal process. The passage of oxygen and, correspondingly of different soluble nutrients, is clearly a complex process.

The importance of trace elements in the nutrients available to growing biofilms was illustrated by Santos (9). Figure 9 shows that in the absence of trace elements there is very little growth but as soon as these compounds were added to the nutrient supply, the growth of the biofilm was very rapid. The small deposit of cells in the first 800 hours is most likely due to cell deposition under the influence of the flow conditions (i.e. no actual growth at all, on the surface). The figure also demonstrates that the nature of the surface influences the growth. The technique used in the work to assess the accumulation of biofilm was based on infrared absorbance; the higher the absorbance the greater the accumulation of biofilm. As in many other studies reported in this chapter, the colonising bacterium was *Pseudomonas fluorescens.*

It is likely that under natural conditions that biofilms grow on rocks and other robust surfaces and that the local pH is near neutral. It is to be expected therefore that excursions of pH will influence growth. In confined spaces microbial activity will affect the local pH. Again using *Pseudomonas fluorescens,* Kaur (10) showed (Figure 10) that as activity increased pH gradually fell. This effect is likely to be more pronounced in the interstices of the biofilm. Here there is a reduced opportunity for the acidic water to be removed and replaced with bulk water. In industrial situations there is the

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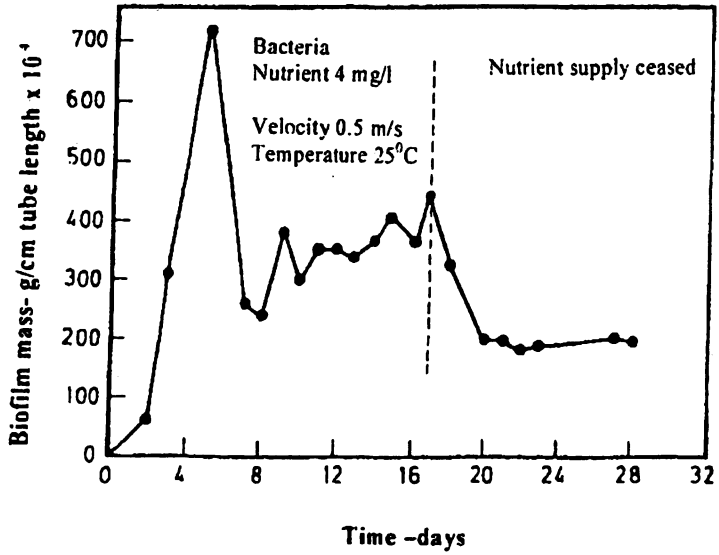

Figure 7. The effect of eliminating nutrients from the flowing water on biofilm growth (7).

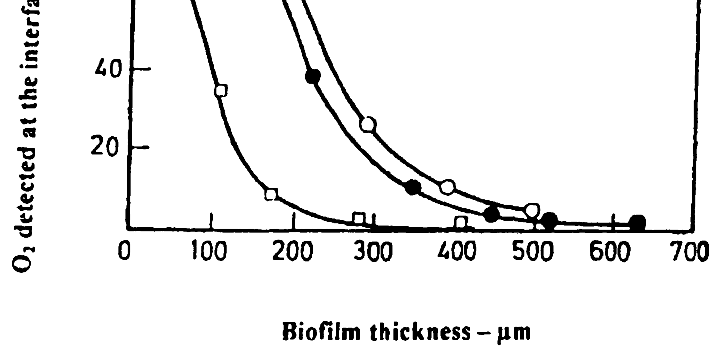

Figure 8. The change in oxygen concentration at the base of developing biofilms (8).

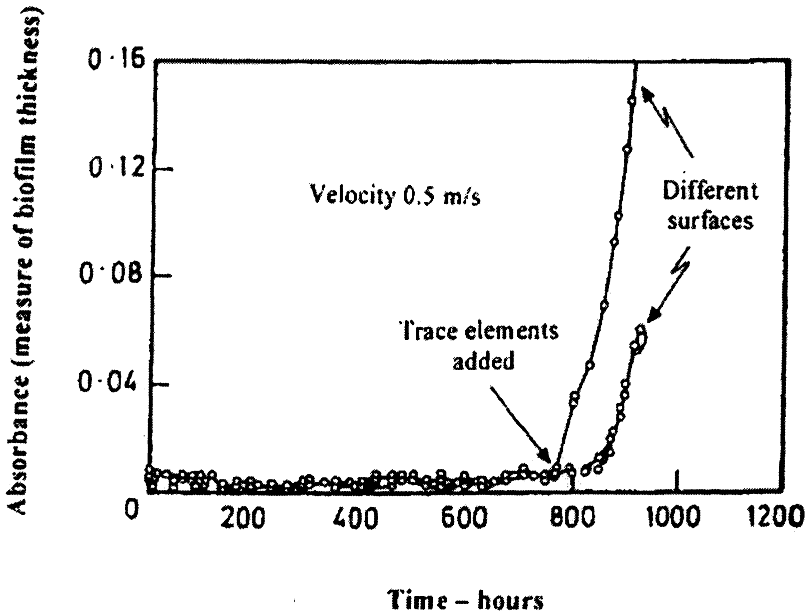

Figure 9. The effect of lack of trace elements on biofilm growth (9).

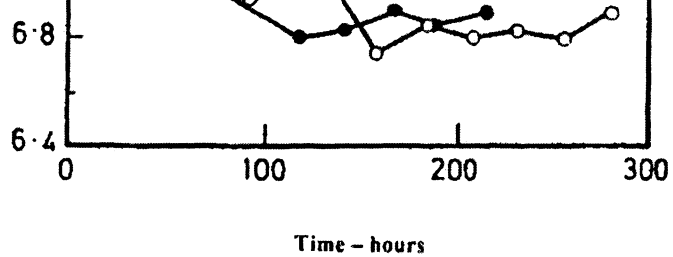

Figure 10. The change in pH as biofilms develop (10).

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distinct possibility that surface corrosion will occcur as a result of this localised change in pH in the adhering deposit of active cells. There may also be the possibility of symbiosis occurring in the biofilm that could affect its quality.

Since microbial activity is affected by temperature it would be expected that temperature will influence the development of biofilms. It was observed (11) that a relatively small increase in the water temperature from 30 to 35°C from which a biofilm was developing (all other operating variables remaining constant), increased the thickness of the biofilm by 70%. The micro-organism involved was *Escherichia coli.* Using the same micro-organism it was demonstrated (12) that as the bulk water flow velocity increased from 0.1 to 0.55 m/s, the difference in biofilm thickness due to temperature became negligible. This effect was probably due to the reduced resistance to heat transfer from the reduced boundary layer thickness as the velocity was increased ensuring a biofilm surface temperature near to the bulk water temperature.

Measurements made on a full scale industrial cooling water system (13), with no biocide present, showed that in the warmer summer months of the year, the biofilms produced were much thicker than those produced in the colder winter months. From midwinter to midsummer the biofilm thickness increased by around 500% (from 75 to 450 μm). The biofilm in this example consisted of a mixed culture.

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The Effect of Surface

The condition of the surface to which the biofilm attaches i.e. rough or smooth, will affect the colonisation and strength of attachment of the micro-organism forming a biofilm. For relatively rough surfaces it is likely that the depth of a crevice is sufficiently large for a cell that happens to deposit there, to be sheltered from removal forces. The opposite is likely to be the situation in respect of smooth surfaces, with the difficulty of cells remaining on the surface long enough to achieve irreversible attachment. Using a circulating system a number of different materials was tested for their support of biofilms (14). Figure 11 illustrates the effects of different material of construction on biofilm growth. Materials tested included "as received" and electro-polished 316 stainless steel, fluorinated ethylene polypropylene and glass. The last three materials show similar behaviour i.e. a lower accumulation of biofilm at the plateau level of the growth curve, compared to the "as received" stainless steel. Accumulation however will be determined, not only by differences in surface roughness but also differences in the physical-chemical properties of the materials (see later).

Biofouling experiments were carried out by Reid et al (15), using three stainless steel bioreactor vessels. The shell of each vessel was manufactured from 316 stainless steel, providing a different micro-roughness (or finish), of the surfaces in contact with the suspension of micro-organisms. The average heights of the roughness, (Ra) as determined by British Standard 1134 for the three

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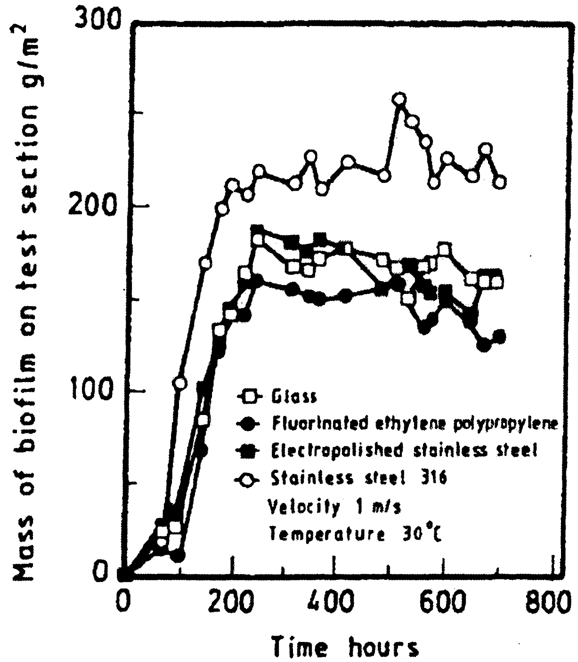

Figure 11. The effect of some different materials on the development of a biofilm (14).

vessels, was 3.8, 2.3 and 0.06 µm. Four species of micro-organism were used in the experiments including *Bacillus subtilis, Penicillium chrysogenum, Saccharomyces cerevisiae and Pseudomonas fluorescens.*. The accumulation of microbial deposits was measured in terms of the protein content of samples taken from the different surfaces, on the assumption that the presence of the protein was the result of biofilm activity. The principal conclusion of the study was that surface finish had a statistically significant effect on adsorbed protein (biofilm) for three out of the four micro-organisms studied. The trend was not apparent for the *Saccharomyces cerevisiae* which was grown and sampled under different conditions. This difference emphasises the importance of maintaining standard conditions when attempting to make comparisons involving biofilms. In two cases the rougher surface (Ra = 3.8) was identified as the source of the significant variation. *Pseudomonas fluorescens* proved to be different. It was the surface roughness of Ra = 2.3 that showed significant variation. It could be considered that these differences are due to the interaction between surface roughness and micro-organism dimensions and possibly interaction with the prevailing conditions. More work on this aspect of biofilm formation would help to clarify the relationship between surface condition and microbial species. It is however, further evidence that similar conditions are essential for comparing the activity of biocides.

Contributing to the attachment of particles including micro-organisms to surfaces are physical, chemical and electro chemical forces. Khilnani (16) has given a useful review of the various mechanisms. Included are the following:

1. van der Waals forces from interacting dipole moments of the components.
2. Capillary force from the surface tension of any adsorbed liquids.
3. Electrostatic forces from any charges on particles and the surface and any charges external to the system of surface and deposit.

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The Structure of Biofilms.

The traditional picture of a biofilm is a solid homogeneous gelatinous mass of living matter. Recent research however using confocal scanning laser microscopy (including the excellent work carried out at Montana State University) has shown that the structure of a biofilm is far more complex. Under water flowing conditions it is shown to consist of a heterogeneous structure consisting of cell clusters separated by interstitial voids and channels (18). Real time video-imaging has also revealed the presence of "streamers" oscillating rapidly under the influence of the flowing water (19). The cell clusters may consist of a single species or contain a mixture of different species (20), perhaps implying that there could be an element of symbiosis present. The channels between the clusters are not well defined and probably result from the orientation of the initial colonisation, the presence of surface conditioning molecules and surface roughness. Extracellular polysaccharides are present around the clusters and to some extent in the channels. The channels are interconnecting and allow flow through the biofilm structure and could therefore, allow the ingress of biocides and nutrients and the removal of waste products from around the cells. Figure 12 gives a diagrammatic picture of the modern concept of biofilm structure (21). It has also been demonstrated that at higher velocities, there is a tendency for cells to be oriented so that they offer the least resistance to the shear forces associated with velocity (17). The mechanism by which this is achieved is likely to be that the cells which happen to be unaligned are removed by the shear forces present. The survivors, the aligned cells, have a reduced chance of being swept away by the flowing water. It is possible however that cell mobility could assist the orientation process.

Basic Laboratory Equipment Requirements

The brief discussion on the effects on biofilm quality of the conditions under which biofilms are formed demonstrate that comparative testing of biocides will only be valid if standard conditions are maintained. Some of the data presented in subsequent Sections confirm this observation.

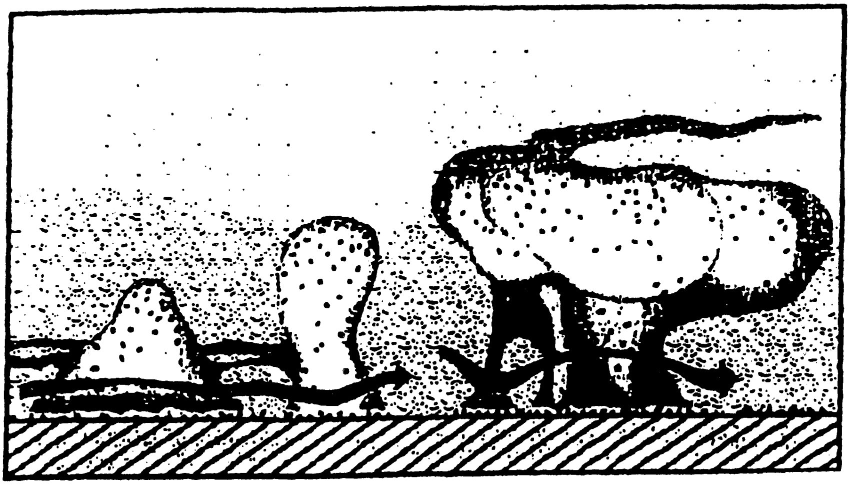

Figure 12. A diagrammatic representation of biofilm structure (after 21).

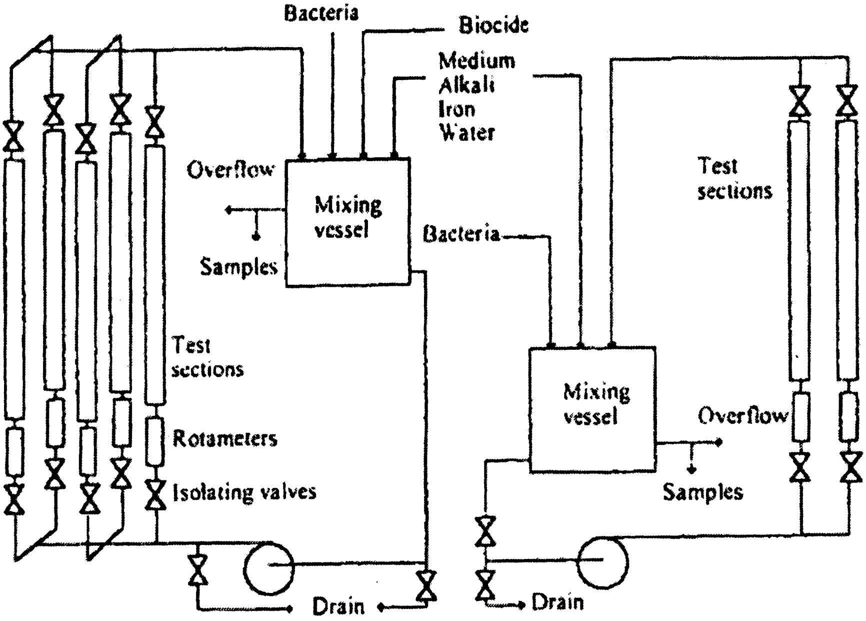
Bacteria
Biocide
Medium
Alkali
Iron
Water
Overflow
Mixing vessel
Samples
Bacteria
Test sections
Test sections
Rotameters
Isolating valves
Mixing vessel
Overflow
Samples
Drain
Drain

Figure 13. Schematic diagram of a pilot plant to test biocide efficacy (22).

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Equipment successfully developed for determining the efficacy of different biocides is shown diagrammatically on Figure 13. The technique involves the circulation of water containing small concentrations of nutrient and contaminated with a fixed concentration of a single species of micro-organism (bacteria). A monoculture is suggested since experience has shown that it is extremely difficult to maintain stable mixed cultures in laboratory equipment, for the long periods of time required by the tests. A fixed composition is essential to obtain reliable data that may be used for comparison purposes. It is necessary also to control the variables that have been discussed earlier.

The contaminating bacteria are grown in a fermenter that is carefully controlled in terms of its operating parameters. Bacteria-laden water from the fermenter is fed at a known rate and composition, into a mixing vessel. Water from the mixing vessel is pumped through vertically mounted test sections on which biofilm is grown. The test sections are mounted vertically in order to avoid any effect due to gravity. After passing through the test sections the water is returned to the mixing vessel. A constant bleed off from the mixing vessel is replaced by an input from the fermenter, together with a fixed nutrient stream and distilled quality water to ensure constant composition feed to the test sections. It is advantageous if the test apparatus can be fabricated in glass to avoid potential complications that might result from the use of metals. If the effect of the material of construction on biofilm production is required then the test sections at least, would need to be made from this material. Glass is in general, corrosion-free and has the added advantage that it gives the opportunity for visible examination. The equipment is sterilised prior to the commencement of an experiment, by passing low pressure steam through the system for at least twenty four hours.

The particular design of equipment and associated experimental design (22) that were used to obtain the data recorded in this chapter, will be discussed in some detail, to illustrate the technology.

Continuous Fermentation

The arrangement of the fermenter system is shown on Figure 14 and provides information on the various components involved. The basic operating conditions are given in Table I.

Table 1. Fermenter Operating Conditions

Parameter	*Magnitude*
Working volume	11 .07 l
Dilution rate	0.02m]/h
Residence time	50h
Temperature	26-28°C
pH	7.0
Viable cell concentration	1-5 x 10^9cfu/ml
Glucose concentration	2-4mg/l
Medium addition rate	3.69 ml/min
Air input	5.50 l/min

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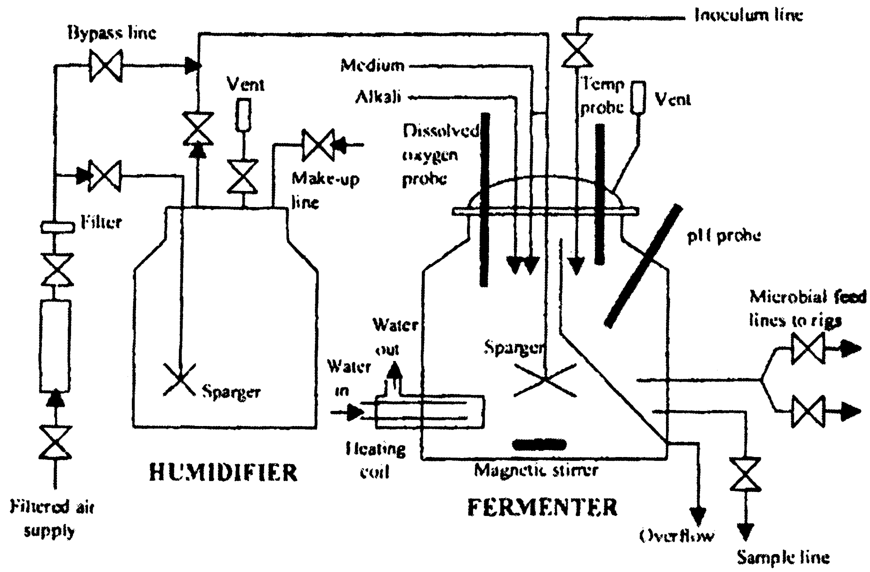
Bypass line
Vent
Medium
Alkali
Dissolved oxygen probe
Make-up line
Filter
Sparger
HUMIDIFIER
Filtered air supply
Water out
Water in
Heating coil
Sparger
Magnetic stirrer
FERMENTER
Overflow
Inoculum line
Temp probe
Vent
pH probe
Microbial feed lines to rigs
Sample line

Figure 14. Detail of the fermenter for producing contaminated bacteria for test purposes (22).

Control of temperature and pH are essential in order to provide the test water at standard conditions, a check on the dissolved oxygen level is also necessary.

A humidifier is required to saturate the air entering the fermenter to prevent water removal from the fermenter during operation. The air is filtered to remove contamination including microbial contamination, that could influence the results of the tests. If this were not carried out the composition of the contents of the fermenter could be variable. Air needs to be injected into the fermenter so that the contaminated water used in the tests, is always saturated with oxygen. This is particularly important when aerobic bacteria are used in the tests, but in any case it is advisable to keep the composition of the water as constant as possible.

The water used in the experiments has to be of distilled water quality i.e. free from dissolved salts and particulate matter. This is achieved by filtration down to 1μm, followed by reverse osmosis or distillation, the former is perhaps the more convenient.

Nutrients

The nutrients are pumped into the system in liquid form. The components are listed in Table 2 and the trace elements in Table 3 All liquids are sterilised in a suitable autoclave before they are introduced into the system. It is important to

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Table 2. Medium Constituents

Component	g/10 litres
$NaH_2PO_4.2H_2O$	10.075
Na_2HPO_4(anhydrous)	55.0
K2SO4(anhydrous)	17.5
$MgSO_4.7H_2O$	1.0
Na_2 EDTA.$2H_2O$	8.303
NH_4Cl(anhydrous)	38.2
Glucose(anhydrous)	50.0
Trace elements solution	10.0ml

Table 3. Trace Elements Solution

Component	*g/ l*
$MnSO_4.H_2O$	13.3
H_3BO_3	3.0
$ZnSO_4$ $7H_2O$	2.0
Na_2 $MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.024

include the trace elements since it has been demonstrated earlier that if they are not present the biofilm will not develop.

Test Sections

It is convenient to use tubes as the test sections on which the biofilm is allowed to develop and as already indicated, it is possible to use tubes made of the material of construction intended to be used for the construction of the full scale plant. In the laboratory pilot plant reported here, the tubes were I m in length with diameters similar to those found in heat exchangers. If the tubes were opaque then the method adopted for the assessment of the accumulation of biofilm was by direct weighing. It was necessary to stop the flow, and remove the tube for weighing. It is difficult to carry out this strategy without disturbing the biofilm or allowing it to lose water by evaporation, and there is the added difficulty of replacing the tube and continuing the experiment. A mean biofilm thickness can be obtained from the data, by assuming that the biofilm has the density of water since it has a high water content of the order of 90-95%, and knowing the internal diameter and length of the tube.

Many experiments however, have been carried out using equipment made of glass with tubes of similar diameters and length. In order to measure biofilm accumulation a special infrared absorption device was developed (23). The reduction in the infrared intensity as the radiation passes through the biofilm, is a measure of the accumulation. The method does not allow the thickness of the biofilm to be obtained directly from the absorption. It would be necessary to correlate the absorbance against thickness obtained by the weighing method; a tedious process. For the assessment of biocide activity however a comparison of the infrared absorption obtained under different conditions is adequate. The principal advantage of this system is that accumulation measurements could be obtained without stopping the flow, continuously if required.

Alternatively test sections could be flat rectangular plates (say 100x20mm) fitted into a suitable housing across which the contaminated water is made to flow. A construction difficulty is being able to fit the test plate so that it does not cause any disruption to the flow. Furthermore there is the problem as with opaque tubes, of restarting the experiment after weighing, without disturbing the biofilm in some way. An alternative method of measurement for assessing biofilm accumulation on flat plates, is to employ an electrical conductivity technique (4). This method allows the biofilm thickness at a particular point, to be measured directly. An average thickness can be obtained by taking a number of readings and obtaining a mean value.

It is possible to use a specially designed tapered test section, rectangular in cross section (24), so that a gradual change in velocity occurs as the flow progresses through the test section. (i.e. data at a series of velocities is obtained at the same time) The same limiting restrictions occur in this method as described for the assessment of data obtained from a metal tube and flat plate test sections.

In order to increase productivity it is feasible to have a number of test sections in parallel operating at different velocities; all other variables being identical in each section. Under these circumstances it would of course be necessary to ensure that the fermenter and the mixing vessel were of adequate size to cope with the likely demand. The control parameters in the test section array are pH, temperature, and dissolved oxygen.

To make comparisons of the effectiveness of different biocides or indeed, to investigate the result of different biocide dosing techniques, it may be necessary to obtain data on the growth of a biofilm under similar operating conditions but without the presence of the biocide. For this reason it would be necessary to have two separate mixing vessels fed from a common fermenter, one mixing vessel without added biocide and the other with the appropriate feed of biocide. A set of separate test sections would also be required to operate under the same conditions as those with added biocide.

It is possible of course, to mount the test equipment close to a source of natural water that is used or could be used, for cooling purposes and to use this water to produce the biofilm on the test surfaces. This adoption of the basic technique could yield useful data from "real" contaminated water. There could

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be difficulty however, in maintaining consistent conditions, particularly in the consistency of the biofilm due to biological changes occurring in the feed water that might prove to be misleading. Nevertheless it could be argued that this was testing closer to the conditions that would be experienced in practice, rather than in the ideal conditions of a laboratory.

Monitoring Bacterial Accumulation on Surfaces.

The usual microbiological methods of assessing micro-organism contents of suspensions may be used, e.g. viable cell counts and ATP measurement but the estimation of the accumulation of micro-organisms on the test surfaces would need to be by a different method. As already alluded to in earlier discussion, the method adopted would to a large extent, be dictated by the design of the test sections and the material from which they were constructed. It is worthwhile discussing these techniques in a little more detail in this section, which is devoted to growing consistent biofilms.

It is a substantial advantage if the development of a biofilm can be measured in situ. Without interruption of the flow conditions. A technique developed at the University of Birmingham involves infrared absorption. In essence the amount of infrared absorbed by the biofilm at a particular point, is a measure of the biofilm thickness at that point (23). The principal drawback of the method is that the test surface has to be transparent to the radiation. For this reason the test section, usually tubular, is fabricated from glass and this could be considered as unsatisfactory where the results of the tests are to be applied to commercial process plant consisting of opaque metal or polymeric materials with different surface properties. However for the testing of biocide efficacy that involves differing dosing regimes with alternative biocides, the data obtained is adequate for comparison purposes. It is possible to construct a calibration curve of infrared absorbed against biofilm thickness, but this is generally unnecessary as comparisons can be made directly using the measured values of absorbance.

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Examples of Data on Biocide Performance

The following examples illustrate the value of testing biocides under carefully controlled conditions to provide consistent biofilms. The micro-organism in these examples is *Pseudomonas fluorescence* unless otherwise stated.

The effect of water velocity with a low concentration of ozone as a biocide, for the removal of a biofilm is shown in Figure 15 (25). As the water velocity is increased the mass transfer of ozone to the biofilm increases due to the lower mass transfer resistance (thinner boundary layer). The biofilms were grown initially before the ozone was applied, at the same velocity as used for the test.

The reason for this was to ensure that there could be no problems of interpretation. The results indicate that the rate of removal of biofilm, at least in the first few hours of ozone application, is very much higher at the higher velocities than at the lower velocity of 0.6 m/s.

The effect of a higher water velocity is confirmed in a series of experiments using different well-known biocides (26). Figure 16 shows the effect of velocity on the effectiveness of a proprietary biocide on the removal of a biofilm The vertical axis "absorbance" is the infrared absorbed by the biofilm, mentioned earlier and is a measure of the biofilm accumulation. It is clear that a higher velocity in conjunction with a biocide is more effective. It is interesting to note that the effectiveness of the propriety biocide persisted for some time after addition had ceased. It is very likely that this biocide was a mixture containing a dispersant that was retained in the biofilm after the addition of biocide had ceased.

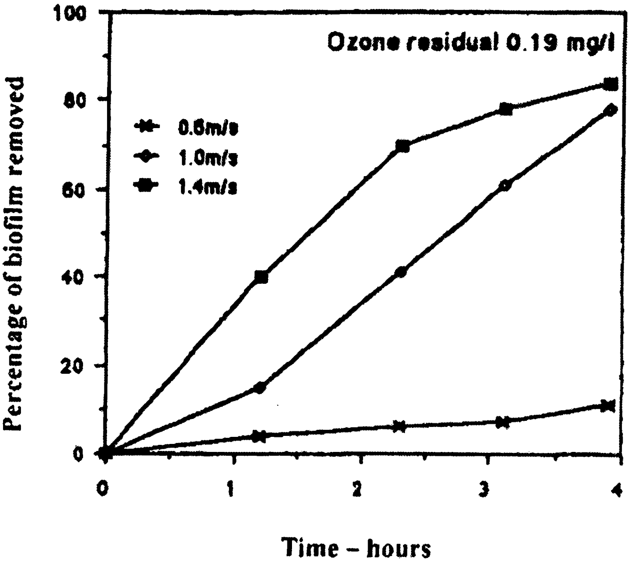

Figure 15. The effect of velocity on the effectiveness of ozone as a biocide (25).

Some recent studies (27) have explored the effects of different dosing strategies using a proprietary biocide containing 2,2 Dibromo-3-nitrilopropionamide. Figure 17 shows that continuous dosing at 100 and 50 mg/l was sufficient to control biofilm formation. A reduction of biocide concentration to 20mg/l showed that there was some biofilm growth as detected by the infrared absorbance. At this level however, the presence of the biofilm could not be seen with the naked eye. A final reduction of biocide addition to 15mg/l resulted in an increase in biofilm attachment, particularly noticeable at the lower water velocity. It is likely that this is a mass transfer effect in that the resistance

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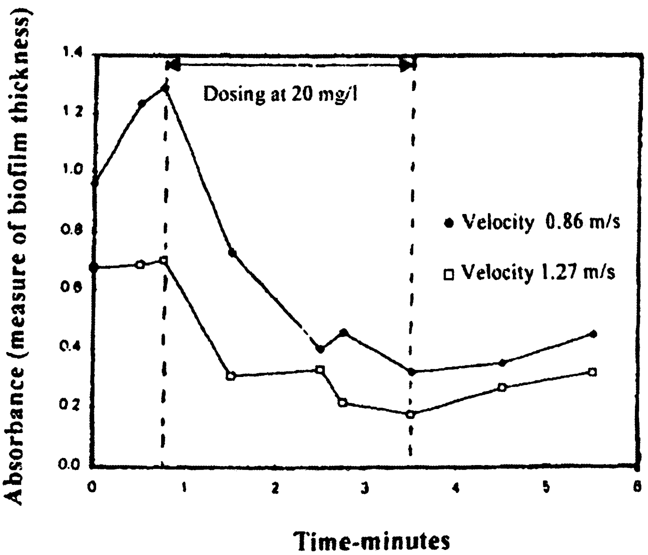

Figure 16. Biofilm removal using 20 mg/l of a proprietary biocide at two water velocities (26).

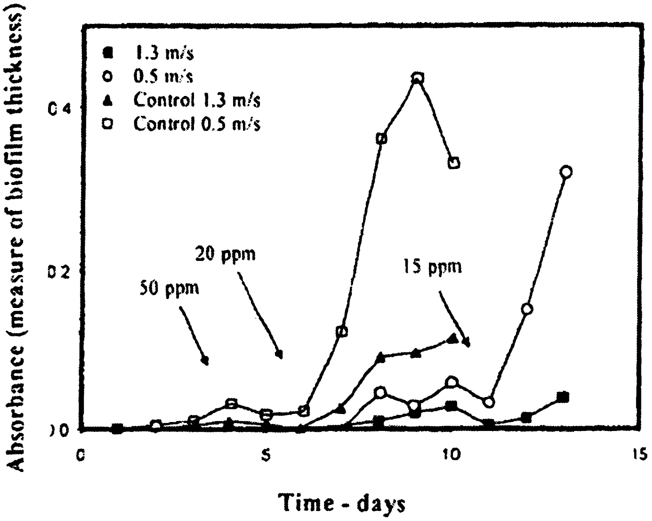

Figure 17. The effect of continuous dosing with a proprietary biocide with reducing concentrations at two water velocities (Reproduced with permission from reference 27. Copyright 2005 Taylor Francis.)

to mass transfer of biocide to the biofilm, is higher at the lower turbulence level corresponding to the lower velocity. At the higher water velocity there was a large measure of control at 15mg/l biocide, reflecting the higher mass transfer rate at the velocity of 1.3 m/s.

An alternative to continuous dosing is shock dosing involving a single burst of biocide over a short period of time on a long-term periodic basis (the usual convenient basis is once a day). As 100 mg/l of biocide used on a continuous basis, was effective this dose was used to investigate shock dosing by injecting over a period of 15 minutes. The result of the test is given on Figure 18 indicating there was some control but this dosing regime was not really very effective,

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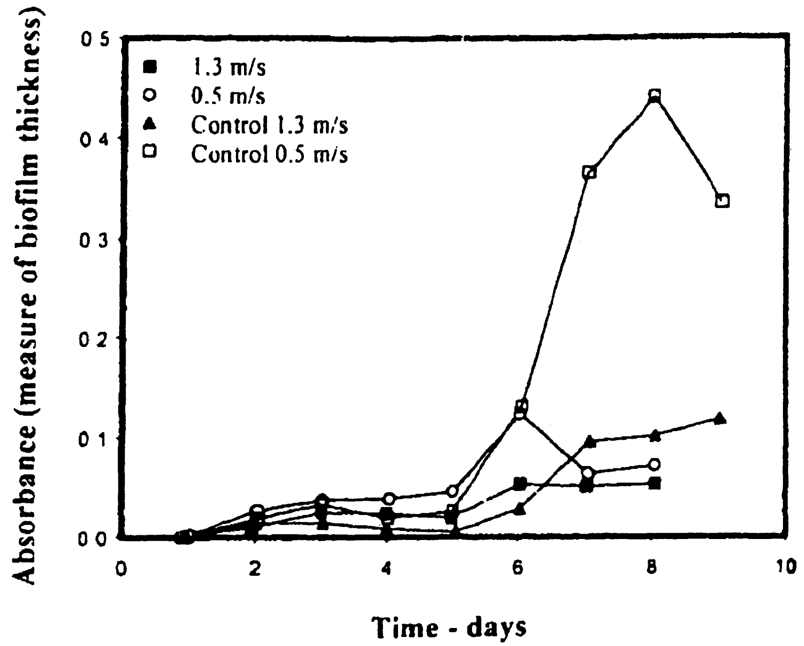

Figure 18. The effect of daily shock dosing with a proprietary biocide at a concentration of 100 mg/l at two water velocities (Reproduced with permission from reference 27. Copyright 2005 Taylor Francis.).

Another method of dosing -pulse dosing- involves injecting biocide for a fixed period of time at regular intervals. Figures 19 and 20 provide data obtained for 4 x 15 minute and 8 x 15 minute dosing regimes per day, with maximum biocide concentrations of 106 and 53.1 mg/l respectively in each pulse. It may be seen that both strategies were successful.

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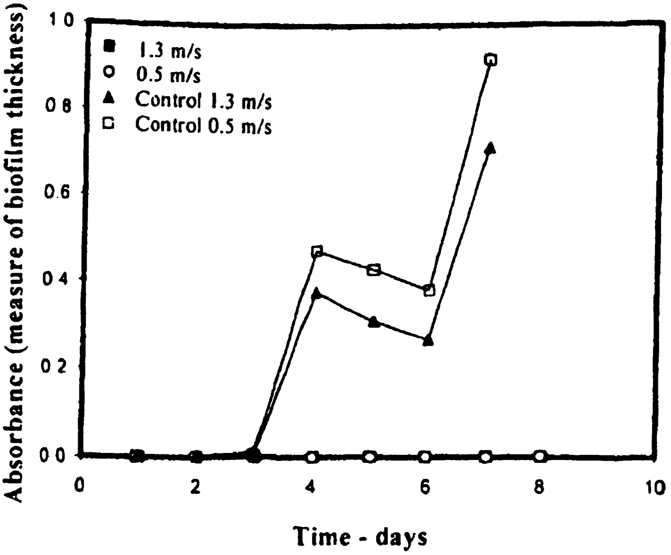

Figure 19. The effect of pulse dosing a proprietary biocide for 15 minutes four times per day at a peak concentration of 106 mg/l (Reproduced with permission from reference 27. Copyright 2005 Taylor Francis.).

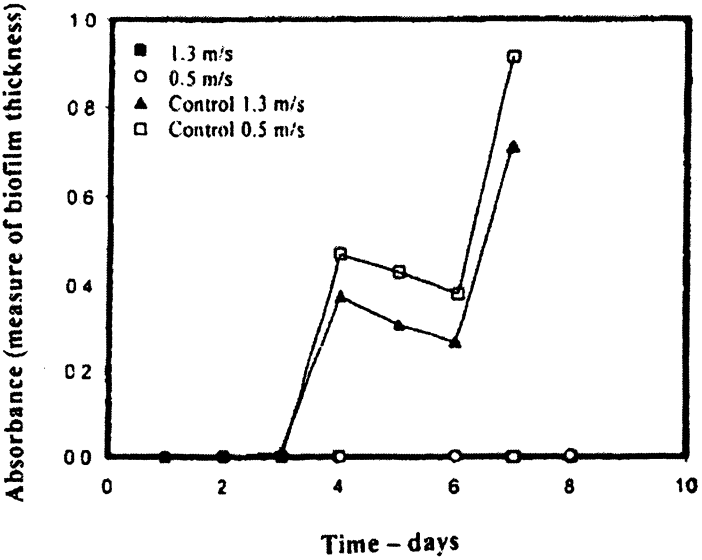

Figure 20. The effect of pulse dosing a proprietary biocide for 15 minutes eight times per day at a peak concentration of 53.1 mg/l (Reproduced with permission from reference 27. Copyright 2005 Taylor Francis.).

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To enhance the performance of biocides with a view to reducing operating costs and with environmental considerations in mind, tests have been carried out using inserts and ultrasound.

Inserts inside tubes have been used to increase turbulence in the fluid flowing through the tube and at the same time reduce the thickness of the laminar sub - layer. From the earlier discussion it will be appreciated that these two effects will improve the effectiveness of an applied biocide. Tests have been carried out using wire wound inserts produced by Cal Gavin, that fit tightly inside tubes (28), thereby not only creating increased turbulence in the bulk flow but disrupting the laminar sub-layer. Both effects would be expected to facilitate biocide activity. Figure 21 presents some of the results obtained in the tests which illustrate the benefit. "high" and "low density" refer to the number of loops per unit length of insert. In simple terms this means that in the former there are more wires per unit length than in the latter. The biocide used in the tests was tetrakishydroxymethyl phosphonium sulphate with continuous dosing of 15mg/l at the beginning of the experiment reduced to 10 mg/l after about 250 hours of testing. Although biofilm growth in the test sections was recorded in the first few hours, this did not continue and disappeared after about 72 hours running. From Figure 21 it would appear that the biofilm was very well controlled with the biocide dose of 15mg/l. Reducing the biocide concentration to 10 mg/l, control was still apparent although there was some growth at the lower water velocity of 0.86 m/s. There was no significant difference between the different insert densities. The absorbance of 2.5 recorded for the low density insert at a water velocity of 0.86 m/s is the maximum limit of the particular monitor used in the experiment, suggesting that the biofilm was much thicker than the monitor indicated.

The use of ultrasound to improve the effectiveness of biocide application has recently been reported (29). The biocide employed was ozone in water, dosing at 2.2 -2.8 mg/l for 3 hours each day. Six glass tubes mounted vertically, were used as the test sections on which the biofilm was grown. Tubes 1 and 2 were fitted at their base with probes producing ultrasound of 20kHz operated at 20% amplitude for one minute three times per day at eight hour intervals. Fig. 22 summarises some of the extensive tests continuously carried out up to 550 hours. It can be seen that a combination of ultrasound and very low concentrations of ozone, is effective in controlling biofilm growth. The biofilm thickness was reduced to about 10μm.

Conclusion

In order to test and make comparisons of the effectiveness of biocides for the control of biofilm growth in flowing water, it is vital that the tests are carried

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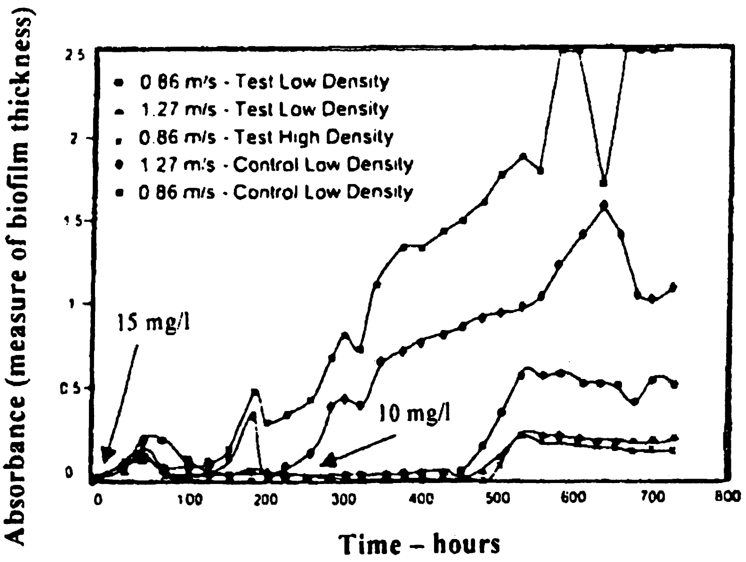

Figure 21. A comparison of biofilm development in the presence of a tube insert with and without a proprietary biocide present (28).

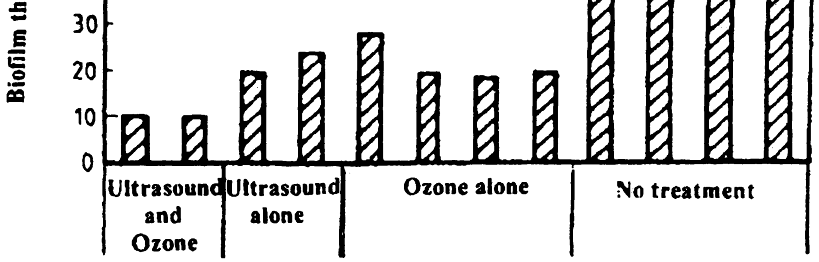

Figure 22. A comparison of biofilm thickness in the presence of ozone, during the application of ultrasound, in combination, and with no biocide or ultrasound treatment (29).

out under carefully maintained identical conditions. As would be expected the extent and quality of biofilms are affected by the conditions under which they develop. It has been demonstrated that there are four groups of variable conditions that are involved. They include the concentration of micro-organisms and the availability and quality of nutrients. The external conditions of water flow velocity, temperature and pH and the quality and substance of the surface on which the biofilm forms also affect biofilm accumulation. Therefore, in any test programme it is vitally necessary to be certain that these variables are suitably controlled. Monocultures of micro-organisms are recommended because it has been found to be extremely difficult to maintain the consistency of systems of mixed species for the length of time required to carry out meaningful tests. Changes to the eco - system will affect the composition of the associated biofilm thereby affecting the quality of the results obtained.

A basic design of a laboratory test facility that has yielded useful results, avoiding common drawbacks, is discussed with examples of the kind of biocide assessment data that may be obtained.

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Chapter 6

Topical Antimicrobials

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In this chapter, antimicrobials commonly used as antiseptics in the hospital, food service, and consumer product settings will be addressed. I will take a systems approach in this effort, presenting skin surface interactions among microorganisms and antiseptic agents. First, a discussion of the skin structure and function will be presented, followed by a discussion of various microorganisms (bacteria, fungi, and viruses) important in skin antisepsis, and finally, a discussion of antimicrobial agents currently available in topically-applied products.

I. Human Skin

Human skin, the largest single organ, normally presents a continuous protective covering over the body, providing a near-impermeable barrier to microorganisms, as well as regulating the moisture levels of the tissues it covers. The skin constitutes about 16 percent of the total body weight, having an area of approximately 1.8m^2 for average-sized adults.[1]

Structure

The skin consists of two general layers, the epidermis and the dermis (Figure 1), composed of cells in varying stages of keratinization.[2] Both layers contain elastic fibers that are responsible for the elasticity and firmness of the

In New Biocides Development; Zhu, P.;
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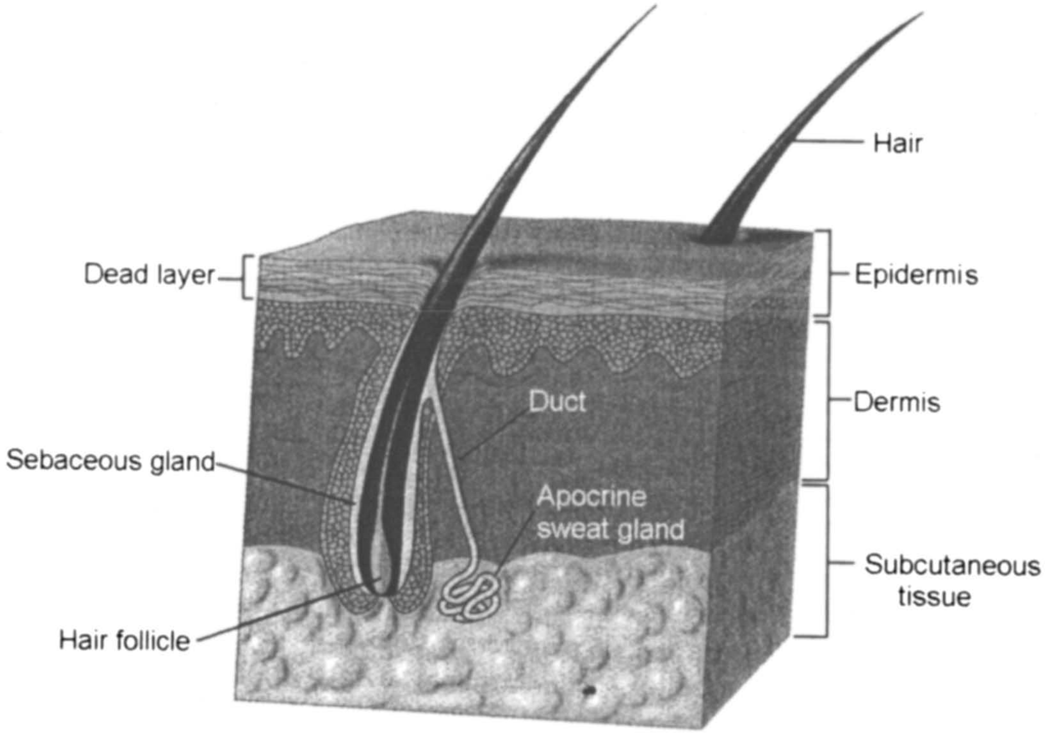
Hair
Dead layer
Epidermis
Duct
Dermis
Sebaceous gland
Apocrine
sweat gland
Subcutaneous
tissue
Hair follicle

Figure 1. Composition of human skin[3]

skin. The epidermidis contains no blood or lymph vessels; however, the inner layers consist of metabolically active cells, strongly bound together by desmosome junctions. Underlying the epidermis is the dermis, made up of connective tissue containing both blood and lymphatic vessels, and varying in thickness in different regions of the body. Human dermal skin comprises two layers with rather indistinct boundaries: the outer papillary layer and the deeper reticular layer. The papillary layer is thin and composed mainly of loose connective tissue, containing fibroblasts, mast cells, macrophages, and migrating white blood cells. The reticular layer is thicker than the papillary layer and is composed of dense, irregularly arranged, connective tissue, containing more collagen fibers and fewer cells than the papillary layer.

The subcutaneous tissue lying beneath the dermis varies in thickness, as well as histological makeup, in different anatomical regions. It contains an intricate vascular network that supplies nutritive, immunological, and coagulative properties. A nerve network connecting various subcutaneous fibers brings sensory stimuli from the skin surface to the brain and conveys certain autonomic motor stimuli from the brain to various specialized involuntary muscle structures.

Let us now look at the epidermis and dermis in a broader perspective.

The Epidermidis

The epidermidis, the outer layer of the skin, is from 75 to 150 μm thick and normally presents an impenetrable barrier to "non-resident" microorganisms, while also providing a suitable environment for a variety of "resident" microorganisms. These resident microorganisms are important in skin ecology, in that they impede epidermal colonization by other potentially pathogenic microorganisms.[1, 2] The epidermis harbors a variety of resident microorganism species with colony counts $< 2.0 \log_{10}$ per cm^2 at dry skin sites to $>6.0 \log_{10}$ per cm^2 at moist skin sites.

The epidermidis comprises several layers that can be visually distinguished from each other. The inner-most layers consist of metabolically active cells strongly bonded by spot desmosome junctions. As can be seen in Figure 1, the epidermidis is stratified and lies on top of the dermis. It is separated from the dermis by the basement membrane, or basal lamina, a continuous and structureless sheet distinct from the cytoplasmic membranes of the adjacent cells. It is supported by numerous extracellular fibrils, which can be regarded as part of the basal lamina, as well as by some of the uppermost dermal cells.[4, 5]

The stratum corneum, the most external component of the epidermidis, consists of several layers of flattened, dead squamous cells containing large amounts of keratin and firmly attached to one another. The intercellular spaces are filled with multiple lipid bilayers, packed so that there is an alternating

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pattern of nonpolar hydrocarbon regions and polar head group regions. The lipids involved are cermides, cholesterol, cholesterol sulphates, and free fatty acids organized together in multilaminated shells. The bilayers consist of straight, loosely-packed, saturated hydrocarbon chains. This highly-ordered rigid structure results in a unique impermeability to many compounds, including water. In some cells, remnants of nuclear membranes can be observed with a light microscope. The most external cells of the stratum corneum are constantly shed as minute skin particles (squames) and, in certain conditions, as visible flakes and sheets (e.g., dandruff). This cell loss is compensated by constant replacement of cells rising from the lower epidermal layers. That is, epidermal cells continuously move toward the skin surface in successive stages of cellular differentiation and finally death, until they are lost to the environment via exfoliation. This exfoliation process provides a constant replacement of nutrients for resident microorganisms colonizing the skin surface. For the most part, the nutrients present on the skin surfaces do not support growth of fastidious, nonresident microorganisms,[6] but the normal skin microorganisms have evolved to survive on the sebaceous secretions within hair follicles and from "sweat" produced by apocrine glands.

The different layers of the epidermidis all contain keratin, but during the process of keratinization, different types of keratin are produced. These are composed of 19 different α-helix proteins with molecular weights ranging from 60,000 to 68,000.[1]

The normal epidermal cell cycle in human skin is 20 – 30 days, depending on the regions of the body. The rate of regeneration of the epidermidis is regulated according to its thickness. Faulty control of proliferation causes a skin disorder called psoriasis in which the rate of basal cell proliferation is greatly increased, and epidermal cell cycles are completed within a week, without complete keratinization.

The Dermis

The dermis, unlike the epidermis which overlays it, contains blood vessels, an intercellular matrix, and fluid and lymphatic vessels that provide the dermal cells nutrients and immunological protection.[4] The human dermis consists of two layers, the boundaries of which are rather indistinct – the outermost papillary layer and the deeper reticular layer. The papillary layer is thin and composed of loose connective tissue, fibroblasts, mast cells, macrophages, and extravasated leukocytes. The papillary layer penetrates into the papillae, and collagen fibers from the papillary region extend through the basal lamina and into the epidermidis. They are thought to have the special function of binding the dermis to the epidermis and are often termed keratinal anchoring fibers. Intercellular substances provide strength and support of tissue and act as a medium for the diffusion of nutrients and metabolites between blood capillaries and the dermal cells in support of cellular metabolism. The papillary layer adheres tightly to the basement membrane of the epidermidis, while the lower surface merges gradually with the reticular layer.

The reticular layer is thicker than the papillary layer and contains varying amounts of fat, often as a function of an individual's sex and age, and the anatomical region of the body. Females generally show a greater amount of fat within the subcutaneous layers than do males. The reticular layer also contains the larger blood vessels and nerves, from which arise various superficial cutaneous branches to supply the skin.[7, 8]

Both layers of the dermis are interspersed with connective tissue consisting of bundles of collagenous, elastic, and reticular fibers grounded in an amorphous, intercellular substance containing various types of histologically discrete cells. An important component of the dermis is its vascular system is the rich network of blood and lymph vessels. Arterial blood brings oxygen and nutrients that are utilized at capillary junctions of the dermal cells. The veinous blood carries away products of metabolism, also collected at the capillary junctions. Additionally, the vascular system carries cells of the immune system to all parts of the dermis.[1, 2, 4]

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Sebaceous Glands

Sebaceous glands are found embedded in the dermis over all areas of the body, except in those areas lacking hairs. These glands open into short ducts that end in the upper portion of a hair follicle. Sebaceous glands arise structurally from undifferentiated, flattened epithelial cells. The cell nuclei gradually shrink, and the cells become filled with fat droplets and burst. As these cells differentiate, sebaceous glands move toward the surface of the skin. The sebaceous gland secretes sebum, together with remnants of dead cells. Sebum is composed of a complex mixture of lipids containing triglycerides, free fatty acids, cholesterol, and cholesteryl esters and is secreted continuously. A disturbance in the normal secretion and flow of sebum is one of the reasons for the development of acne.[7]

Sweat Glands

The sweat glands are widely distributed in the skin of the face in the form of simple, coiled, tubular structures. The fluid secreted by these glands contains mainly water, sodium chloride, urea, ammonia, uric acid, and proteins. The constant evaporation of sweat requires heat, which is withdrawn from the capillaries surrounding the gland; this loss of heat contributes to the thermoregulation of the body.[7, 8] Most skin microorganisms are associated with the apocrine (sweat) glands and sebaceous glands, because their secretions are rich in urea, amino acids, salts, lipids, and lactic acid. The skin surfaces are normally acid, with a pH range of 4 to 6.

II. Microorganisms and the Skin

Bacteria, fungi, viruses, and other parasites are the causative agents of microbial disease. However, in evaluating topical antimicrobial products such as surgical scrubs, preoperative preparation solutions, and healthcare personnel handwashes, bacteria provide the indices most commonly used in estimating antimicrobial effectiveness.

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Etiology of Infectious Diseases

For an infectious disease to spread, an encounter, an entry, a spread, multiplication, and damage by the microorganisms must occur. To complete this progression of infection, the microorganism must breach the host's defenses, both active and passive.

Immune System

In normally functioning immune systems, the body is able to generate a variety of "immune system" cells and antibody molecules that are capable of detecting ("recognizing") and eliminating an apparently infinite variety of foreign substrates and microbial forms that include viruses, bacteria, and fungi.[9, 10]

Microbial Nutrition

At first glance, it would appear that the body offers a variety of rich mediums for the support of microorganisms. Body fluids such as plasma contains sugars, vitamins, minerals, and other substances on which bacterial and fungi can subsist. However, for microorganisms other than normal flora, life in or on the body is not so simple. For example, if fresh blood plasma is incubated with challenge microorganisms, microbial growth is generally nonexistent or sparse. This is because antimicrobial substances such as lysozyme and the various molecular constituents of the complement system are inhibitory.[11, 12]

Bacteria require free iron for the synthesis of their cytochromes and other enzymes, and this appears to be a limiting factor for their growth in the body. Plasma and most other body fluids contain very little free iron, probably due to its avid binding to a wide range of proteins.[13] In fact, the body actually sequesters iron to defend itself against bacterial multiplication.[14] When a sufficient number of microorganisms has been detected within the body, iron-binding proteins literally pour into plasma and other tissue fluids, as the body strives to limit the availability of free iron to bacteria.

The range of nutritional requirements of microorganisms that are found as normal microbial flora is a reflection of their ecological niches. For example, *Staphylococcus epidermidis*, the predominant skin surface-colonizing bacterium, requires several amino acids and vitamins that are commonly found on the skin surfaces.[14] However, microorganisms common in both soil and water are much less fastidious. They can meet their organic requirements from simple carbon compounds that are widely available in the body, as well as the natural environment. Both *Escherichia coli* and *Pseudomonas* spp. are examples of bacteria that can thrive on very nutrient-poor media.[15]

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Physical Factors

Physical factors on and within the body also affect microbial multiplication. Important physical factors include temperature, osmotic pressure of fluids, and humidity.[16] Microorganisms that are normal inhabitants of the body, or those that can live on the skin, tend to have much more limited tolerance to physical changes than do those also found commonly in the environment, like *Pseudomonas* sp.[17]

Endogenous Microorganisms of the Body: The Normal Flora

It is important, in studying the role of topical antimicrobial products, to be familiar with the microorganisms that compose the normal flora. These are important, particularly in the surgical environment where normal flora may be introduced into a surgical wound by surgical staff or contaminatively from the patient's own skin and opportunistically produce an infection.

The body contains dozens of different bacterial species, as well as numerous different viruses and fungi,[18] the most common sites of colonization being the digestive tract (mouth and large intestine), the respiratory tract (nose and oropharynx), the female genitalia, and the skin surfaces.[12] Normally, the flora are commensal microorganisms that cause no disease in human hosts, because a dynamic balance exists between the host's immunological defenses and the microorganism's ability to cause disease.[19] If that balance is upset, the normal flora can take advantage of the situation. Hence, in major or minor surgical wounds, species such as *Staphylococcus aureus* and *S. epidermidis* can establish an infection.[20]

Opportunistic infectious disease is increasingly prevalent, due particularly to medical advances that prolong life in patients suffering disease (e.g., cancer, diabetes, etc.) that would have killed them in earlier times, or treatments that, themselves (e.g., cancer therapy), compromise the immune system.[19, 21] Opportunistic infections are also common following invasive diagnostic procedures and treatments.

The Bacterial Cell

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Among life forms, two basic cell types are found: eurcaryotes and prokaryotes.[16, 17, 22, 23] Eukaryotic cells are unit structures of higher plants, animals, fungi, most kinds of algae, and certain single-celled organisms such as protozoa. Prokaryote cells, which generally are simpler structurally than the eukaryotes, are the form of all types of bacteria and blue-green algae. They do not contain a nucleus bounded within a membrane but, instead, have a nuclear filament, replicated nonmitotically.[24, 25] Procaryotic cells are typically enclosed in a rigid cell wall containing a unique constituent, muramic acid. Bacteria are classified morphologically into one of three categories: cocci (spheres), bacilli (rods), or spirals.[26] The cocci and bacilli are the most common.[25] Bacteria are distinguished further as being Gram-positive or Gram-negative, using the Gram staining method.[25] Devised by Hans Gram, the Gram stain ranks among the most useful and important stains for identifying bacteria, allowing one to distinguish between bacteria that exhibit similar morphology.[12] As a result of differences in the structure and chemistry of their cell walls, Gram-positive bacteria stain purple,[25] and Gram-negative bacteria stain red. There are various other staining techniques useful in differentiating bacteria, including acid-fast staining, capsule-staining, flagella-staining, metachromatic granule-staining, spore-staining, and relief-staining.[25] With staining, one can determine microscopically the sizes and shapes of the bacteria, the great majority of which exhibit one of three basic morphologies – coccus, bacillus (rod), or spirals. In older cultures, bacteria may exhibit variations on or blending of these shapes and of their staining properties, where bacterial cell structures become weakened and break down.[27]

Bacterial Cell Structures

Most bacteria are enclosed in multi-layered cell structures that include a cytoplasmic (plasma) membrane, a cell wall with associated proteins and polysaccharides, and in some, protective capsules and slime layers that help protect against the host's immune system, particularly phagocytosis.[14, 16, 24] Many also bear external filamentous appendages (flagella and pili) that function in locomotion, tissue attachment, and/or specialized reproductive processes.

Generally speaking, the bacterial cell wall is a rigid structure that is thought to be an evolved structure that allowed bacteria to tolerate a wide range of environment conditions. It encloses and protects the inner "protoplast" from physical damage (e.g., conditions of low external osmotic pressure). Internally, bacteria are relatively simple prokaryotic cells.[25, 26] Major cytoplasmic structures include a central fibrillar chromatin network surrounded by an amorphous cytoplasm containing ribosomes. Cytoplasmic inclusion bodies – energy storage granules – vary in chemical composition, depending upon the bacterial species, and in number, dependent upon the bacterial growth phase and environmental conditions (e.g., temperature). Some cytoplasmic structures (e.g., endospores) are found in a few bacterial species. Typical Gram-positive and Gram-negative bacterial cells, which differ primarily in cell wall organization, are shown in Figure 2.

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Bacterial Size and Form

Medically important bacterial species vary from approximately 0.4 to 2.0 μm in size and appear under the light microscope as spheres (cocci), rods (bacilli), or spirals (vibros or spirochetes).[16, 17] Cocci are found singly, in pairs as diplococci, in chains (e.g., *Streptococcus* spp.) or, depending upon division planes, in tetrads or in grape-like clusters (e.g., *Staphylococcus* spp.). Bacilli vary considerably in length and width, from very short rods (coccobacilli) to long rods with lengths several times their diameter. The terminal ends of bacilli may be gently rounded, as in enteric organisms such as *Salmonella typhi,* or squared, as in *Bacillus anthracis.* Long sequences of bacilli that have not separated into single cells are called chains, while long, thread-like bacilli are generally referred to as filaments. Fusiform bacilli, found in the oral and gut cavities, are tapered at both ends.[24] Curved bacterial rods vary from small, comma-shaped or mildly helical forms with only a single curve, such as *Vibrio cholerae,* to longer spiral (spirochete), multiply-coiled forms, such as species of *Borrelia, Treponema,* and *Leptospira.*

Cell Envelope

Bacterial cells are generally bounded by the cell envelope, an integrated structure of varying complexity and generally of several layers.[16, 17, 27] The bacterial cell envelope commonly includes a cytoplasmic membrane, the

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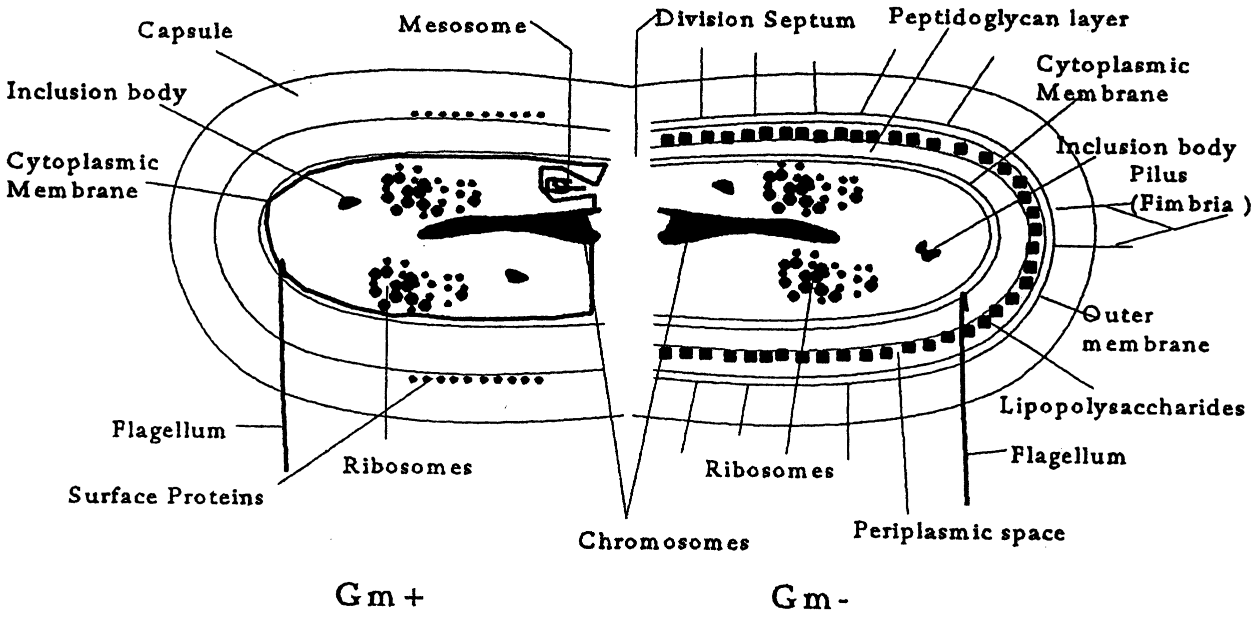
Capsule
Mesosome
Division Septum
Peptidoglycan layer
Cytoplasmic Membrane
Inclusion body
Cytoplasmic Membrane
Inclusion body
Pilus (Fimbria)
Outer membrane
Lipopolysaccharides
Flagellum
Surface Proteins
Ribosomes
Chromosomes
Ribosomes
Periplasmic space
Flagellum
Gm +
Gm -

Figure 2. Cross-section of Gram-positive and Gram-negative cells[28]

overlying cell wall, specialized proteins or polysaccharides, and various outer adherent materials. This multilayered structure constitutes 20% or more of the procaryotic cell's dry weight[22] and contains transport sites for nutrients, receptor sites for specific bacterial viruses, sites of host antibody and complement reactions, and components often toxic to the host.

Among bacteria, the nature of the cell envelope varies considerably in structural complexity and differentiation. The simplest envelopes are found among the members of the genus *Mycoplasma*, which possess only a cell membrane, which averages about 7.S nm thick.[24] Because the cell membrane structure has the dual role of "cell wall" and "cytoplasmic membrane," and does not contain the peptidoglycans found in true cell walls, mycoplasmas possess limited cellular strength.

The envelope structure of Gram-positive bacteria is more complex. In addition to an inner cytoplasmic membrane, having the appearance in cross-section of a "double-track," or two-rail fence membrane, when viewed under the electron microscope, the cell is surrounded by a thick amphorous cell wall, composed of peptidoglycan and teichoic acid. The cell wall, viewed sectionally, does not exhibit the layered appearance of the cytoplasmic membrane and generally is from 15 – 50 nm in thickness.

The cell envelope of Gram-negative bacteria is the most complex. The inner layer of the envelope is a cytoplasmic membrane, structurally similar to that of Gram-positive cells. The overlying cell wall is composed of a second (outer) membrane often appearing wrinkled or with a "raisin-like" appearance.[26] Closely apposed to the inner surface of this membrane, not often visible as a separate layer, is a peptidoglycan layer. The Gram-negative wall is thinner than that of Gram-positive species, usually averaging about 10 – 15 nm across and containing considerably less peptidoglycan.

Capsules and Slime Layers

The virulence – the ability to cause disease – of certain pathogenic bacteria often is related directly to their production of a capsule.[14, 17, 18] For example, virulent strains of *Streptococcus pneumoniae* produce capsules that protect them from phagocytosis by neutrophils and macrophages. It has been demonstrated that loss of the capsule-forming ability produces a loss of virulence and increased ease of destruction by host phagocytes. Although capsules and slime layers are composed of similar "gels" that adhere to the outer cell wall, slime layers are more easily "washed off" than are capsules.[24]

Most bacteria, possibly all species found in the environment (wild types), are surrounded by a layer of the gelatinous, poorly-defined and poorly stainable material that has been termed the capsule, slime slayer, glycocalyx, or biofilm.

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Biofilms provide a very important function in terms of bacterial populations, for they provide a protective barrier from antimicrobial therapy. Additionally, the biofilm matrix enables bacteria to communicate chemically, a process termed quorum-sensing. The communication appears to serve as a regulatory mechanism for metabolic rates, sharing genetic information, in terms of drug resistance, and cellular division.[27] The term, capsule, generally is applied to the material surrounding a single cell, while the slime layer is more commonly used to describe the matrix that envelopes a microcolony or group of bacterial cells, but these distinctions are somewhat arbitrary.[24]

Fungi

Fungi, unlike bacteria, are eucaryotic cells that contain at least one nucleus, a nuclear membrane, an endoplasmic reticulum, and mitochondria.[16, 30] Fungal cells are much larger than bacteria, are closer structurally and metabolically to cells of higher plants and animals[31] and are enclosed in a rigid chitinous cell wall. Unlike most members of the plant kingdom, fungi are non-photosynthetic.[32] The natural habitats of most fungi are water, soil, and decaying organic debris, and most are obligate or facultative aerobes.[31, 33]

Fungi may exist as single oval cells (yeasts), that reproduce by budding, or of long tubular strands (hyphae) that exhibit apical growth and true lateral branching.[16, 17, 28] Branching intermingled and often fused, over-lapping hyphae constitute the mycelium that forms the visible mold colony.[31, 32] Reproduction may be vegetative (asexual) via variously specialized germinal cells called conidia, or it may consist of elaborate specialized structures that facilitate fertilization, protection and dissemination of the resultant spore. A conidium may be a simple fragment of a hypha, or it may be produced from variously specialized structures called coniophores. Spore-bearing structures may be simple lateral branches of the hyphae, or they may be constructed into large reproductive bodies such as mushrooms and bracket fungi, which protect the spores as they develop and facilitate their dispersal at maturity.

Yeasts

Yeasts are single cells, usually spherical to ellipsoidal ranging from 3 to 15 μm in diameter.[17, 30] Most reproduce by budding, but a few do reproduce by binary fission.

After growth on agar media (24 – 72 hours), yeasts tend to produce colonies that are pasty and opaque-looking, generally from 0.5 to 3.0 mm in diameter. A few species have characteristic pigments, but most are cream-colored. Using

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microscopic and colonial morphology, it is difficult to distinguish species, so nutritional studies and specialized media must be used.[31, 32]

Molds

The molds are growth forms that are multi-cellular and filamentous and appear in colonies.[31, 32] The colonies consist of branching cylindrical tubules varying in diameter (2 to 10 μm) termed hyphae, which grow by apical elongation. The mass of intertwined hyphae that accumulates and constitutes the visible mold is termed the mycelium. The hyphae of some species are divided into cells by cross-walling (septa) that form at regular, repeating intervals during filamentous growth and, in some, the septa are penetrated by pores that permit flowing of cytoplasm, but not organelles. Most common species produce asexual reproductive cells, the conidia, on specialized structures called conidiophores. Molds are extreme opportunists, tending to grow well on a tremendous variety of substrates found in the environment and on most laboratory culture media.[31, 32]

Thermal Dimorphism

Most fungi exist only as a mold or a yeast form, but some, including several important pathogens in humans, are capable of growing in a yeast or in a mold form, depending on temperature.[16, 17, 30] At about 35 – 37°C, such species grow in the yeast phase, but at lower temperatures (20 – 30°C), they grow as molds. Although this commonly is referred to as thermal dimorphism, available nutrients, carbon dioxide levels, cell density, age of culture, or some combination of these factors also can induce a shift in growth form.

Viruses

Viruses are a unique life form quite distinct from procaryotic or eucaryotic organisms.[16, 17] They are smaller than bacteria and fungi and are obligate parasites within the cells of other organisms. They consist of a single type of nucleic acid – DNA or RNA, either single- or double-stranded – encased by a shell (capsid) variously structured and composed primarily of protein. The capsid of some viruses is further enclosed by a unit membrane composed of lipids and glycoproteins (so-called "spike proteins").[17, 28, 34] Viruses do not reproduce by binary fission, because they lack the intracellular components necessary to produce macromolecules. Instead, viruses "capture" and redirect the organelles of a host cell to synthesize new virus components, which then

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autoassemble within the host cell into virions, the complete viral particle.[16, 17, 25, 26]

Virions range in size from about 20 to 300 *nm*, with some helical forms as large as 80 × 1000 *nm*. Viral nucleic acid is encased by a capsid that generally takes the form of either a helix or an icosahedron, although some viruses take neither form, and are termed complex viruses. The nucleic acid/capsid is referred to as the nucleocapsid. The capsid, itself, is composed of single polypeptide chains, termed structural units, which may aggregate to form the multiple polypeptide units termed capsomeres, or combine directly to form the helix-form capsids.[26, 35] The capsomere combine to form the more complex icosahedral capsids. The virion, a mature infectious virus particle, may consist of the nucleocapsid (capsid containing nucleic acid) alone, or may be surrounded by an envelope of lipids and glycoproteins acquired during the process of "budding" through the cytoplasmic membrane of the host cell.[24, 35] Viruses are generally grouped into morphological categories based on virion structure (icosohedral, helical, or complex), whether the virion is naked or enveloped, and the form of nucleic acid (single- or double-stranded DNA or RNA).

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III. Topical Antimicrobial Compounds

In this section, we will discuss various antimicrobial compounds commonly used as the active ingredients in topically-applied products found in the medical, food service, and consumer (personal hygiene) markets. Those of primary interest are the iodine complexes (aqueous iodophors and tinctures), aqueous formulations of chlorhexidine gluconate, tinctures of chlorhexidine gluconate, tinctures of iodine, triclosan, parachlorometaxylenol (PCMX), alcohol formulations, and quaternary ammonium species.[36, 37]

Iodine Complexes

Iodine, a nonmetallic element discovered in 1812 by Courtois, a French chemist, one of the halogens with an atomic weight of 126.9. Iodine, in its pure form, is only slightly soluble in water. By adding alkali to form triiodides and polyiodides, its solubility is greatly enhanced. Iodine also dissolves well in polar organic solvents – alcohol, ketones, and carbonic acids – and takes on a brown color. In apolar solvents (benzene, hydrocarbons), it is violet in color. Iodines used as skin antiseptics are generally divided into three groups, according to solvent complexing with the iodine: 1) pure aqueous solutions, 2) alcohol solutions, and 3) iodophoric solutions. Alcoholic solutions (tinctures) of iodine are used primarily as skin antiseptics prior to a catheter placement into a vein or artery and for some preoperative skin preparation procedures.

By far, the most common form of iodine used as a topical antimicrobial is the iodophor.[38] Iodophors are complexes of elemental iodine (triiodine) linked to a carrier having several functions: (1) increased solubility of the iodine in aqueous solution; (2) provision of a reservoir for sustained release of the iodine; and (3) reduced equilibrium concentrations of free iodine. Commonly used carriers are neutral polymers, polyacrylic acids, polyether glycols, polyamides, polysaccharides, and polyalkalines.

The iodophor, povidone iodine, a compound of 1-vinyl-2-pyrrolidione polymer with available iodine ranging between 9% and 12% (United States Pharmacopeia XXIII), is extremely common in preoperative skin preparation products.[39] The chemistry of aqueous solutions of iodophors is complex.

The amount of free molecular iodine is highly relevant to the antimicrobial effectiveness of iodophors. The differing composition of pharmaceutical additives (e.g., detergents and emollients), which have iodine-complexing properties, as well as the ratio of free iodine to total iodine in various proprietary formulations, can result in great differences in the concentrations of free molecular iodine available for binding. Hence, the antimicrobial index of importance for a product is the "total available iodine."[40] For example, povidone iodines containing relatively low concentrations of free molecular iodine may not be capable of autosterilization.[41] *Pseudomonas* spp. and *Burkholderia cepacia* reportedly have been isolated from iodophor concentrates, in which apparently, the bacteria are protected by slime coats.[42]

Although iodines play a fundamental role as skin antiseptics, preoperative skin preparations, surgical scrub solutions, preinjection preparations, and preinsertion preparations for venous/arterial catheters, they also have been used successfully as therapeutic agents to treat wound infections, including those encountered in burn patients. They also have found use in disinfection of medical equipment, such as sutures, catheters, scalpels, plastics, rubber goods, brushes and thermometers, and as antimicrobial treatments for drinking water, swimming pool water, and waste water.

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Range of Action

Iodines provide excellent immediate (within 10 minutes of drying on skin surfaces) antimicrobial action against a broad range of viruses, Gram-positive and Gram-negative bacteria, and fungi.[41] It should be noted, however, that exposure times and concentrations of available iodine required vary (Table 1).[41]

In topical application to skin surfaces (e.g., hands and body surfaces in the inguinal, abdominal, antecubital, subclavian, and femoral regions), iodophors

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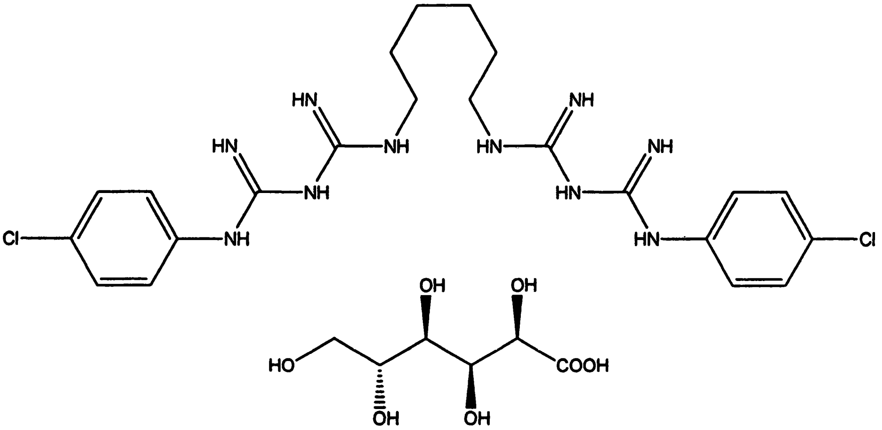
Table 1. Some recommended applications for Iodine-based antimicrobials

Scope of Application	Concentration	Conditions	Exposure time	Disinfective Result	References
General Germicidal action	1:20,000	Absence of organic matter	1 min.	"most bacteria are killed"	Goodman & Gillman, 1980
	1:20,000	Absence of organic matter	15 min.	"wet spores are killed"	Goodman & Gillman, 1980
	1:20,000	Absence of organic matter	15 min.	"will destroy all vegetative forms of bacteria"	Goodman & Gillman, 1980
Disinfection of skin	1% tincture	--	90 sec.	"will kill 90% of the bacteria"	Goodman & Gillman, 1980
	5% tincture	--	60 sec.	"will kill 90% of the bacteria"	Goodman & Gillman, 1980
	7% tincture	--	15 sec.	"will kill 90% of the bacteria"	Goodman & Gillman, 1980
	1% aqueous I_2-solution	Skin of hands	20 min.	"inactivation of rhinovirus"	Carter et al., 1980
	2% aqueous I_2-solution	Skin of hands	3 min.	"inactivation of rhinovirus"	Carter et al., 1980

and tinctures of iodine providing at least 1% available iodine demonstrate effective immediate and persistent antimicrobial properties.[41] However, it should be noted that, in general, neither provides residual antimicrobial action.[43, 44]

Chlorhexidine Gluconate



Chlorhexidine – 1, 6-di (4′ -chlorophenyldiguanido) hexane, a bisbiguanide – was first synthesized in 1950 by ICI Ltd in England as an outcome of research on anti-malaria agents.[45] It is most commonly found in products as a salt of gluconic acid, and is referred to as chlorhexidine gluconate, or CHG. CHG was found to have high levels of antimicrobial activity and, fortunately, low toxicity to mammalian cells.[45, 46] CHG has a strong affinity for binding to the skin and mucous membranes. As a result, it has been used in topical antimicrobial products for treating acute and chronic wounds, preoperative skin-prepping, surgical handwashes, healthcare personnel handwashes, and preparation of mucous membranes, particularly in dentistry, where its binding properties provide extended antimicrobial activity. CHG also has been used as a preservative, including for ophthalmic solutions, and as a disinfectant of medical instruments, and nonporous environmental surfaces.

CHG is cationic and generally is compatible with other cationic molecules, such as the quaternary ammonium compounds, certrimide, and benzalkonium chloride.[45] Some nonionic detergents, although not directly incompatible with CHG, may neutralize the antimicrobial properties of CHG. CHG is not compatible with inorganic anions, except in very dilute concentrations, and also may be incompatible with organic anions present in soaps containing sodium lauryl sulphate, and with various pharmaceutical dyes.[38, 40, 45]

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The antimicrobial activity of CHG is pH-dependent, with an optimal use range of 5.5 – 7.0, which is also the body's usual range of pH (5.0 – 8.0). However, the relationship between antimicrobial effectiveness of CHG and pH varies with the microorganism.[45] For example, CHG's antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* increases with an increase in pH, but the reverse is true for its activity versus *Pseudomonas aeruginosa.*

The antimicrobial activity of CHG against vegetative forms of both Gram-positive and Gram-negative bacteria is pronounced.[45, 47] It generally is inactive versus to bacterial spores, except when they are exposed at elevated temperatures. The mycobacteria – "acid-fast" bacteria reportedly are inhibited, but not killed by CHG in aqueous solutions. A variety of lipophilic viruses and enveloped viruses (e.g., herpes virus, HIV, influenza virus) are rapidly inactivated by exposure to CHG. Finally, certain fungi, particularly those in the yeast phase, are sensitive to CHG.

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At relatively low concentrations, CHG exerts bacteristatic effects on bacteria. At higher concentrations, CHG demonstrates rapid bactericidal effects. However, the precise mechanisms of these effects vary from species to species, and as a function of concentration of CHG.[45, 47] In general, it is known that the microbicidal effects occur in a series of steps related to both cytological and physiological changes, which culminate in the death of the cell.[45, 46] CHG is known to have an affinity for bacterial cell walls and is adsorbed onto certain phosphate-containing bacteria cell wall structures. By this process, the CHG is thought to penetrate the bacterial cell wall by overcoming the bacterial cell wall's molecular exclusion mechanisms. Once penetration has occurred, the CHG is attracted to the cytoplasmic membrane, resulting in leakage of low molecular weight cellular components (e.g., potassium ions) out of the membrane and inhibition of membrane-bound enzymes such as adenosyl triphosphatase. Finally, the cell's cytoplasm precipitates, forming complexes with phosphated compounds, including ATP and nucleic acids.

As a rule of thumb, bacterial cells carry a total negative surface charge.[45, 46] It has been observed that, at sufficient CHG concentrations, the total surface charge rapidly becomes neutral and then positive. The degree of the shift in the charge is directly related to the concentration of CHG, but reaches a steady state equilibrium within about five minutes of exposure. The rapid electrostatic attraction between the cationic CHG molecules and the negatively-charged bacterial cell surface contributes to the rapid reaction rate, that is, the rapid bactericidal effects exerted by CHG.

Products containing CHG at antiseptic concentrations (0.5 – 4%) demonstrate a high degree of antimicrobial effect, both -static and -cidal, on vegetative phases of Gram-positive and Gram-negative bacteria, but it have little sporicidal activity.[45] Although there have been concerns that prolonged exposure to CHG would lead to reduced microbial sensitivity, even the development of resistant strains of bacteria, this concern has not been verified, even after prolonged and extensive use.[45, 46] There is no evidence that plasmid-mediated antimicrobial resistance, particularly common in Gram-negative bacteria, has developed. This has been borne out in studies of common indicator species such as *Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens,* and *Proteus mirabilis.* Although several researchers have reported a reduced sensitivity to CHG among certain methicillin-resistant strains of *Staphylococcus aureus* (MRSa) at clinical-use concentrations, this concern has not been substantiated. MRSa strains are as susceptible to CHG as are methicillin-sensitive strains (MSSa).

Because viruses have no synthetic properties of their own, the action of CHG is restricted to the nucleic acid core or the viral outer shell. Some viral coats consist of protein and others, lipoprotein or glycoprotein. The outer envelopes that enclose the viral shell of some viruses are composed mainly of lipoprotein.

In general, product containing higher concentrations of CHG (4% to as low as 0.5%) provide excellent immediate and persistent antimicrobial activity with the added benefit, when applied to skin repeatedly over time, of good residual efficacy.[45, 46] Products with CHG at lower concentrations (less than 0.5%) provide antimicrobial action comparable to that of PCMX and triclosan.

Products based on CHG, due to its residual antimicrobial properties, are useful for full or partial bodywashes prior to elective surgery. If the product is used once per day over the course of at least 3 to 5 days, the resident microbial populations are reduced by about 3 $\log_{10}$.[19] Hence, when a person undergoes surgery, the remaining microbial populations residing on the proposed surgical site have been significantly reduced, leaving far fewer microorganisms to be eliminated by preoperative prepping procedures.

Currently, there has been much interest in alcohol plus CHG.[36, 37] These tincture of CHG products likely will prove to be highly effective as preoperative skin-preps in non-moist sites, as surgical hand cleansers, and as preinjection, and pre-insertion skin preparations for arterial/venous catheterization. Preparations of alcohol/CHG combine the excellent immediate antimicrobial properties of alcohol with the persistence properties of CHG to provide a clinical performance superior to either alcohol or CHG, alone.

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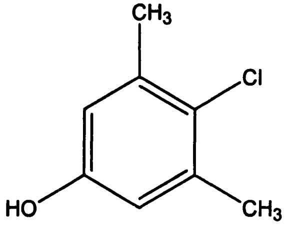
Parachlorometaxylenol (PCMX)

PCMX (4-chloro-3, 5-dimethyl-phenol) is one of the oldest antimicrobial compounds in use, dating back to 1913. A halophenol, it has not been widely used as a surgical or presurgical skin preparation because of its relatively low antimicrobial efficacy.[48] Because of the absence of substantive data in 1972, the FDA did not designate PCMX as a safe and effective antimicrobial, and it was



not formulated at the time for medical purposes such as surgical scrubs, preoperative skin preps, or healthcare personnel handwashes. The initial evaluations from the FDA listed PCMX as a Category III product, meaning that there were not enough data to recognize it as both safe and effective as a topical antimicrobial.[48]

Studies since 1980 have demonstrated PCMX to be safe for human use. After this determination, a number of companies became interested in developing products based one PCMX for use as a topical antimicrobials, and later studies have demonstrated many of these to be effective antimicrobial products.

Current over-the-counter formulations demonstrate varying degrees of antimicrobial efficacy, depending upon the formulation. Generally, PCMX products achieve fair to good immediate effects and fair persistent effects, but like iodophors, they provide no residual antimicrobial activity. Currently, PCMX products are mainly formulated for healthcare personnel handwash applications. These are effective in removing transient (contaminant) microorganisms from the hands and have low skin irritation properties. As a caveat, however, higher levels of PCMX, through having improved antimicrobial activity, often are irritating to the skin, with repeated use.

Alcohols

Alcohol antiseptic agents are widely used in medical, food service, and consumer products. They are particularly popular for their "near-instant" antimicrobial properties. The antimicrobial effects of alcohols are highly dependent on the concentration used (generally, 60 to 95%), as well as the moisture level of the microbial environment treated. The short chain, monovalent alcohols – ethanol, isopropanol, and n-propanol – are probably the most effective for skin disinfection, because they are highly miscible with water, have low toxicity and allergenic potential, are fast-acting, and are microbicidal, as opposed to microbistatic.

Alcohols are generally considered nonspecific antimicrobial agents, due to their multi-level mechanisms of action, including protein coagulation and

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denaturation, disruption of cytoplasmic membrane integrity, leaching of cell wall lipids, and disruption of cellular metabolism. Alcohols denature the cell wall and lyse the cytoplasmic membrane, releasing the intracellular contents. Protein coagulation is observed at optimum alcohol levels (60 – 95%) in the presence of water in the cell wall and cytoplasmic membrane, and, given that proteins are associated with enzymatic activity, cell metabolic function is destroyed.

Alcohols are generally inactive against bacterial spores. And, although there is much controversy in the literature concerning the efficacy of alcohols against viruses, there appears to be a general agreement that enveloped, lipophilic viruses are more susceptible to inactivation by alcohols that are the "naked" viruses. Lastly, the fungicidal properties of alcohols vary among fungal species, but in general, alcohols demonstrate a relatively high degree of mycocidal/-static activity.

Although ethanol, isopropanol, and n-propanol provide excellent immediate antimicrobial activity, they have virtually no persistent or residual antimicrobial properties.[48] However, because their immediate killing effects are so pronounced, the rebound effect on bacteria is longer than most surgical procedures, so that concern is muted. Alcohols have been shown to provide adequate results as healthcare personnel hand cleansers, over-the-counter instant hand sanitizers, presurgical hand cleansers, and preinjection skin preparations in removing or killing transient microorganisms, but when used at strengths of 70% and greater, they tend to be drying to the skin, resulting in significant irritation.

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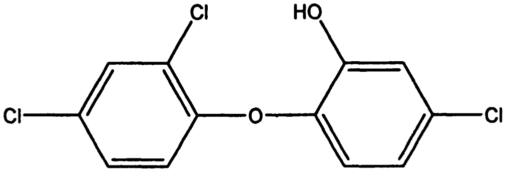
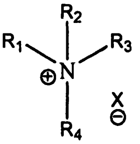
Triclosan



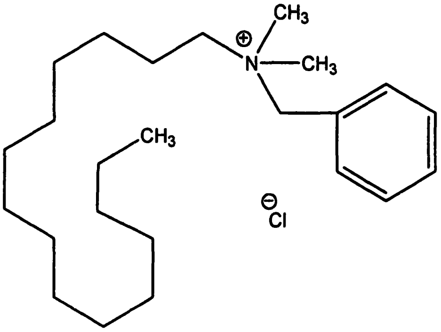
Triclosan (5-chloro-2-[2,4-dichlorophenolxy]phenol), like PCMX, provides varying degrees of antimicrobial efficacy, depending upon the specific formulation and the species of microorganism tested.[36, 37] Triclosan has been formulated for a wide range of applications, and products based on it are currently used as healthcare personnel handwash formulations, in the food industry to cleanse workers' hands, and in consumer market lines, including hand soaps, toothpastes, shower gels, and body cleansers. Triclosan, like

PCMX, provides fair immediate and persistent effects, but no residual action. However, Triclosan has come under repeated attack for being used excessively in over-the-counter products (OTCs), such as hand and body soaps, dishwashing solutions, hand and body lotions, and toothpastes, for example, and even cutting boards. It is thought that a real danger is present in microbial resistance to Triclosan. Furthermore, studies indicate that it accumulates in the environment.

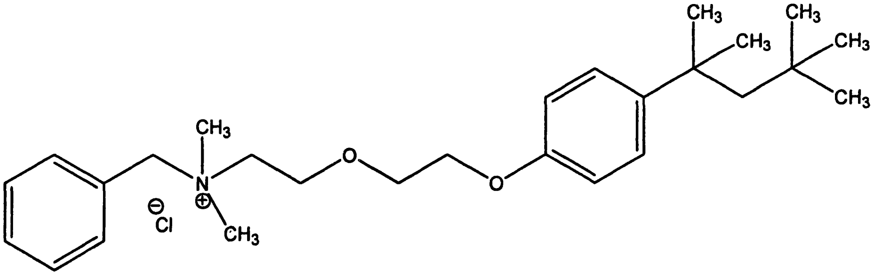
Quaternary Ammonium Compounds (QACs)



General form of QACs, R_1-R_4= alkyl or armatic substutents, X=Cl^-



Benzalkonium chloride



Benzethonium chloride

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Although quaternary ammonium compounds (QACs), such as benzalkonium chloride and benzethonium chloride, have been formulated in products that show varying results as healthcare personnel handwash formulations, quat-based products play only a minor role in the topical antimicrobial market. They do, however, occupy a unique niche in the world of antimicrobial compounds in their use as preservatives. Rather than being a single, well-defined chemical entity, as is the case with the antimicrobially active ingredients we have reviewed, QACs are composed of a diverse, eclectic collection of substances that share a common molecular structure containing a positively charged nitrogen atom covalently bonded to four carbon atoms. This carbon/nitrogen structure is responsible for the name of these antimicrobial compounds and also plays a primary role in determining their chemical behavior.

The first reports of quaternary ammonium compounds with biocidal activity appeared in 1916. Since that time, QACs have grown in popularity and have been utilized extensively as active ingredients in many types of products, including household cleaners, institutional disinfectants, skin and hair care formulations, sanitizers, sterilizing solutions for medical instruments, preservatives in eye drops and nasal sprays, mouthwashes, and even in paper processing and wood preservatives. As a group, QACs are effective across a broad spectrum of microorganisms, including bacteria, certain molds and yeasts, and viruses. However, the specific activity of QACs is as diverse as their range of chemical structures. QAC antimicrobial effectiveness is highly formulation-dependent, because many kinds of compounds can affect QAC activity. Some reduce the QAC efficiency, while others may synergize activity to expand the spectrum of affected microorganisms. This fact has led to some confusion and apparent contradiction in the published literature as to the actual effectiveness of QACs in their role as antimicrobials.

In addition to their antimicrobial activity, QACs also behave as surfactants, assisting with foam development and cleansing action. They also are attracted to the skin and hair, where small amounts remain bound after rinsing. This contributes to a soft, powdery feel to the skin, unique hair-conditioning effects, and persistent activity against microorganisms. These various attributes and multifunctional roles of QACs appeal to formulators and are responsible for their incorporation into many consumer products.

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Conclusion

By far, the most common and effective topical antimicrobials are alcohols, povidone iodine, and chlorhexidine gluconate, and alcohol-based combinations of the latter two. PCMX and Triclosan frequently raise questions from regulatory agencies as to why they exist, because, by themselves, they have not been proven effective.[49]

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Combinations				
	Activity			
Compound	Immediate	Persistent	Residual	Application
Alcohol and CHG	High	High	--	precatheter skin preparations; surgical site preparations
Alcohol and Zinc Pyrithione	High	High	--	precatheter skin preparations; surgical site preparations
Alcohol and Iodophors	High	High	--	precatheter skin preparations; surgical site preparations
4% CHG (aqueous)	Medium	High	High	Surgical scrubs; preoperative skin preparations; healthcare personnel handwashes
2% CHG (aqueous)	Medium	High	High	Surgical scrubs; preoperative skin preparations; healthcare personnel handwashes
Iodophors (1.0 – 7.5%)	Medium	Medium	None	Surgical scrubs; preoperative skin preparations; healthcare personnel handwashes
Parachlorometaxylenol (PCMX)	Medium to Low	Medium to Low	None	Healthcare personnel handwashes
Quats	Medium to Low	Medium to Low	None	Healthcare personnel handwashes
Triclosan	Medium to Low	Medium to Low	None	Healthcare personnel handwashes
Alcohol	High	None	None	Healthcare personnel handwashes; surgical scrubs; consumer products

In New Biocides Development; Zhu, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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Chapter 7

Preparation of Phthalaldehydes by Hydrolysis of Aromatic *gem*-Tetrabromides

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Even though many methods have been used for aldehyde syntheses, few of them can be applied for the preparation of aromatic 1,2-dialdehydes, e.g. orthophthalaldehyde. Hydrolysis of 1,2-bis(*gem*-dihalomethyl)benzene is a good option because of its general applicability and the availability of the material by bromination of ortho-xylene derivatives. The hydrolysis by fuming sulfuric acid followed by work up with water has been evaluated. A novel workup procedure with solid sodium bicarbonate could significantly increase the yield. This method was tested for various compounds.

Some aldehydes are important in the field of biocides and disinfectants[1-2]. Formaldehyde has been used as biological preservative for a long time. However, it has become faded out due to its carcinogenic and volatile properties. Glutaraldehyde is another good disinfectant that has been in use for several decades. But it is disfavored because of its volatility, and irritating and unpleasant smell. *Ortho*-phthalaldehyde (OPA) has been found a superb high level disinfectant. Not only does it avoid the carcinogenicity and volatility issues, but shows a fast mycobiological efficacy as well. OPA-like compounds are a fairly new class of compounds being developed as biocides[3, 4, 5, 6]. There is a general need for new germicidal compounds for disinfection or sterilization.

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The properties of OPA-like compounds must be considered when the preparation procedure is designed. In addition to the general properties of aromatic aldehydes, these compounds have special chemical properties related to the formation of the five-member ring. OPA is fairly stable in normal conditions. However, it is susceptible to some extreme conditions, such as oxidation,[7] reduction[8, 9, 10] and high basic[11] environment, as shown in Figure 1[12].

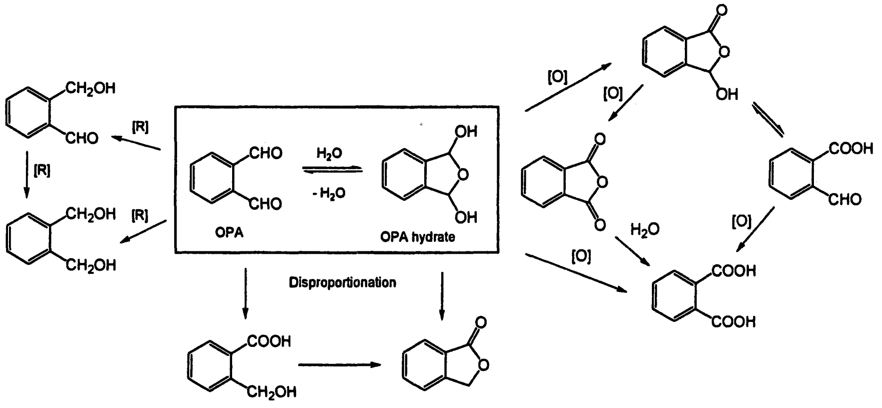

Figure 1. Possible byproducts via Redox or disproportionation reactions of OPA

OPA is more susceptible to oxidation than normal aromatic aldehydes such as benzaldehyde. The oxidation in aqueous solutions could undergo through the cyclic hydrate formation and 2-carboxybenzaldehyde has been found the major degraded product in OPA solutions. Another easy reaction of OPA is the intramolecular disproportionation of the aldehyde groups. This reaction is significantly facilitated by the formation of cyclic hydrate and can go on under mildly basic conditions.

Numerous methods have been used for the preparation of aldehydes. Due to the special properties of OPA-like compounds, few of the methods can be applied for the preparation of these compounds. The oxidation of primary alcohols[13] and reduction of carboxylic acid derivatives with special reagents are classic choices for simple aldehydes preparation. Pyridinium chlorochromate (PCC), pyridinium dichromate (PDC)[14], oxalyl chloride /DMSO (Swern)[15] can selectively oxidize primary alcohols to aldehydes. Preliminary trials with these oxidants gave no desired products or very low yields probably because of the formation of the benzo five member ring. Reductions of acyl chloride or ester with DIBAL-H[16] or $LiAlH(OBu\text{-}t)_3$[17,18] are commonly used too. Hydroboration of alkynes with disiamylborane (Sia_2BH) followed by peroxidation give a good yield of aliphatic aldehydes[19], but is not applicable for aromatic aldehydes.

The electrophilic substitution of arenes, such as Reimer-Tiemann, Rieche, Vilsmeir reactions, could be used for the preparation of aromatic aldehydes. However, they are not applicable for *ortho*-phthalaldehyde derivatives due to the strong directing effect of the aldehyde group present on the benzene ring.

The ozonolysis of alkenes followed by reduction is an efficient method for the preparation of aldehydes. Aromatic version of ozonolysis has been carried out successfully for naphthalene to produce *ortho*-phthalaldehyde in industrial scale. Due to regioselectivity issues, it is not practicable for the preparation of substituted *ortho*-phthalaldehyde.

Among the aldehyde synthetic methods [20, 21, 22], only a few can be used for the synthesis of OPA-like compounds. For example, the hydrolysis of *gem*-dihalides could give aldehydes under various conditions [23, 24, 25]. In this case no reductant or oxidant is involved. This method was used for the preparation substituted *ortho*-phthalaldehydes (Figure 2). The substituted α,α,α′,α′-tetrabromo-*o*-xylene was reacted with fuming sulfuric acid at room temperature. Then the mixture was worked up at controlled temperature. Consideration of OPA properties, especially those for the synthesis of substituted *o*-phthalaldehyde by the hydrolysis of *gem*-dihalides, is crucial in designing synthetic strategy since (1) fuming sulfuric acid involves extreme reaction or workup conditions (2) each dialdehyde has different chemical stability and (3) each tetrabromide requires different hydrolysis conditions. An innovative new approach was reported for the reaction condition control in the synthesis of some OPA-like compounds in order to minimize side reactions and maximize yields. The yield could be significantly improved by consuming fuming sulfuric acid with solid bicarbonate prior to the addition of ice water.

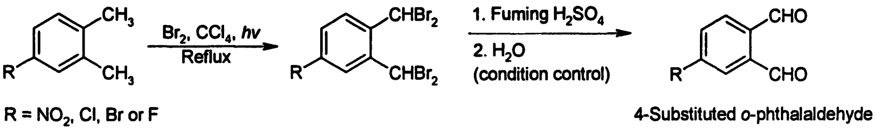

Figure 2. General two-step synthesis of 4-substituted o-phthalaldehydes by bromination/hydrolysis

Bromination of *o*-Xylene Derivatives

The bromination of substituted *o*-xylenes with liquid bromine could be carried out in CCl_4 under tungsten lamps. The HBr generated from bromination was trapped with a Na_2CO_3 solution. The bromine addition rate was adjusted in order to control the concentration of the bromine in the solution. Additional

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bromine was added if the color of the solution became lighter, or colorless. The reaction was allowed to proceed for 2-6 hours. Most of the CCl_4 was removed at normal pressure by distillation at up to 130 °C. Then, a series of methanol were added to the residue to azeotropically remove residual CCl_4 by distillation at up to 130°C. The remaining solvent was removed at 40 °C with a rotary evaporator at about 10 mmHg to produce a solid. The solid was recrystallized in hexane to yield substituted 1,2-bis-dibromomethyl-benzene. The bromination of various substrates is summarized in Table 1.

Table 1. Summary of bromination conditions for substituted 1,2-bis-dibromomethyl-benzene

Substituent	Bromination Time (h)	Purification	Isolated Yield %
4-Cl	6	Crystallization	72.4
3-Cl	45	Crystallization	71.8
4-Br	2	Crystallization	72.4
4-F	6	Crystallization	NA
4-NO2	6	Crystallization	NA

Hydrolysis

Mark et al[26] described the preparation of polyhaloaralkyl (halosulfonyl) sulfooxonium inner salts compounds by the reaction of benzal halides with sulfur trioxide. The oxonium salts were hydrolyzed to afford the target aldehydes. They also mentioned that the aldehyde products of oxonium salt hydrolysis might be oxidized to the corresponding carboxylic acid derivatives.

Li et al[19] reported a similar approach for the preparation of aromatic polyaldehydes, including OPA, by the hydrolysis of *gem*-dibromides. Aromatic polyaldehydes $C_6H_{6-n}(CHO)_n$ ($n = 2, 3$) were prepared by the hydrolysis of corresponding $C_6H_{6-n}(CHBr_2)_n$ with fuming or concentrated H_2SO_4. We improved this process to synthesize 4-chloro-OPA, 4-bromo-OPA, and 4-nitro-OPA. Table 2 comparatively lists the yields obtained for Li's process, and the improved synthesis processes with either reduced acid, or using $NaHCO_3$. The processes are described below.

Hydrolysis Procedure 1

In Hydrolysis Procedure 1, substituted 1,2-bis-dibromomethyl-benzene powder was mixed with 12 mole equivalents of fuming sulfuric acid and stirred

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at room temperature for about 1 h. During the hour the powder was dissolved and the solution gradually became dark brownish. The brownish solution was gradually added to crushed ice with stirring so that the temperature of the solution was controlled. Then the solution was allowed to gradually warm to room temperature, and extracted with ethyl acetate. The organic phase was timely extracted with 5% Na_2CO_3 and brine. The resultant organic phase was dried with anhydrous Na_2SO_4. The lingering solvent was removed at 40 °C by rotary evaporator at about 10 mmHg to obtain a yellow solid. The yellow solid was recrystallized in hexane to yield substituted-OPA as crystals.

Hydrolysis Procedure 2

In hydrolysis Procedure 2, the substituted 1,2-bis(dibromomethyl)benzene was mixed with 12 mole equivalents of fuming sulfuric acid. The mixture was stirred until all bromides are dissolved. Eight mole equivalents of solid sodium bicarbonate powder were added while the mixture was being stirred in an ice bath. After the mixture stopped bubbling, ice was slowly added for the hydrolysis. The remaining processes were the same as Procedure 1, giving the desired dialdehyde.

The synthesis of 4-bromo-OPA and 4-fluoro-OPA followed the similar procedures as described for 4-chloro-OPA. The oil 4-fluoro-OPA was purified by silica column chromatograph (hexane: ethyl acetate = 2:1). Others were purified by recrystallization. Key reaction and workup conditions are summarized Table 2.

Table 2. Yield comparison of three reaction/work up conditions

Product	Process	Equivalents of H_2SO_4	Equivalents of $NaHCO_3$	Isolated Yield (%)
OPA	Reference method [19]	28.4	0	79
	With controlled acid	12	0	86
	With added $NaHCO_3$	12	8	91
4-Chloro-OPA	Reference method [19]	28.4	0	66
	With controlled acid	12	0	88
	With added $NaHCO_3$	12	8	88
4-Bromo-OPA	Reference method [19]	28.4	0	17
	With controlled acid	12	0	88
	With added $NaHCO_3$	12	8	92
4-Nitro-OPA	Reference method [19]	28.4	0	2
	With controlled acid	12	0	28
	With added $NaHCO_3$	12	5.5	44
		12	8	48

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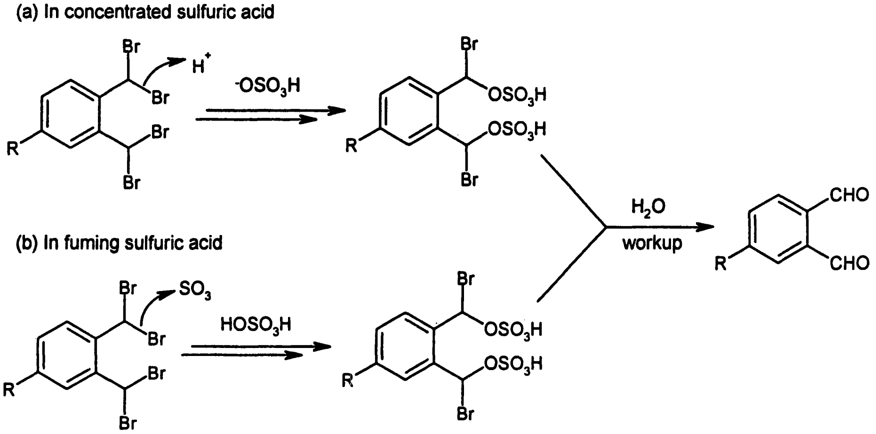
The fuming sulfuric acid was a complex media and contained about 20% SO_3. Since hydrolysis in concentrated sulfuric acid need about 18 h at boiling temperature while only about 1 h at room temperature in fuming sulfuric acid, the reactions must take different mechanisms in these two cases. The key difference might be the first step attacking. SO_3 in fuming sulfuric acid is a strong Lewis acid and could cleave the C-Br bond more easily than proton in concentrated sulfuric acid. The observation that the reaction went much easier in fuming sulfuric acid than in concentrated sulfuric acid[18] indicates that Route b dominates. Considering Mark et al's work using liquid SO_3,[26] a dual mechanism is also possible.



Since fuming sulfuric acid could oxidize the intermediates and the aldehyde products, tests of its effect on product 4-bromophthalaldehyde were performed. When fuming sulfuric acid (22.0 g) was poured into the flask loaded with 4-bromophthalaldehyde (4.26 g) at room temperature, the material became dark and the temperature automatically increased due to the oxidation. A large amount of black tar material was found after work up, indicating severe reaction at elevated temperatures. On the other hand, if 4-bromophthalaldehyde (2.583 g) was slowly added and dissolved in fuming sulfuric acid (14.29 g) cooled with an ice bath, a clear solution was formed. After stirred for one hour with the ice bath, the solution was divided into two portions. One portion was added slowly added to about 300 g of crushed ice and white solid was recovered. The other portion was warmed up to room temperature and stirred for one more hour. Then it was added slowly to about 300 g of crushed ice and white solid was similarly recovered. GC-MS and TLC showed no change occurred from 4-bromophthalaldehyde in either case, indicating that 4-bromophthalaldehyde was stable during these processes. Therefore, temperature seems to be the key factor for 4-bromophthalaldehyde oxidation by fuming sulfuric acid.

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4-Bromophthalaldehyde is fairly stable under workup cooled with an ice bath, but its oxidation by fuming sulfuric acid is fast at above room temperature. Probably the oxidation by cooled fuming sulfuric acid undergoes through the reaction of the intermediate.

To reduce the heat released during the workup, an improvement has been discovered by controlling the amount of fuming sulfuric acid. For example, 4-bromophthalaldehyde yield was increased from 17% to 18% when the fuming sulfuric acid was reduced from 28.4 equivalents to 12 equivalents (Table 2). Significant yield improvement was achieved for all the four target products in table 2. However, this approach may have its limitation since sufficient acid is needed for the complete formation of the desired intermediate.

The key discovery of this research is the addition of solid $NaHCO_3$ before the addition of water. Added $NaHCO_3$ at low temperature neutralizes the excess amount of sulfuric acid. This approach widely increased the yield of all the products tested especially for that of 4-nitro-OPA. There are a few additional advantages of this additional step employing $NaHCO_3$. Although minimization of sulfuric acid is useful way to avoid the formation of by product, insufficient (or diluted) sulfuric acid would lead to lower yields due to the incompleteness of the conversion of the bromides to the intermediate. With this approach, the amount of excess sulfuric acid becomes a less concern since $NaHCO_3$ may help to neutralize the extra amount anyway.

It is important to note that stronger bases should be avoided in placed of $NaHCO_3$. A few attempts of using stronger bases all led to lower yields. Such bases include sodium carbonate, sodium hydroxide, potassium carbonate, calcium hydroxide, calcium carbonate and triethylamine. This is for large extent, due to the intramolecular Cannizarro reaction that lead to the formation of alcohol and acid (then lactone). It was found that some sensitive systems such as 3-chloro-OPA even undergo Cannizaro reaction with $NaHCO_3$, while OPA undergoes only slow Canniramo reaction even in 50% NaOH.

Conclusions

An art of condition balancing in synthetic aldehyde chemistry is described. The yields of aromatic dialdehydes by hydrolysis of *gem*-dibromomethylarenes with fuming sulfuric acid may be significantly improved by (1) adding solid $NaHCO_3$ before workup under cooled conditions or (2) minimizing the amount of sulfuric acid in the reaction. The greatest relative improvements were observed for 4-bromo-OPA and 4-nitro-OPA. These reaction conditions also minimized the formation of possible by-products, especially during the course of synthesizing those sensitive dialdehydes. These findings may find wide application in the fine chemical industry.

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Chapter 8

Efficacy and Mycobactericidal Action of Aldehydes: Structure–Activity Relationship

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The aliphatic di-aldehyde glutaraldehyde (GTA) and the aromatic di-aldehyde *ortho*-phthalaldehyde (OPA) are used for the high-level disinfection of medical devices because of their broad spectrum microbicidal efficacy. Mycobacteria represent a particular challenge to disinfection as they are intrinsically resistant organisms to many microbicides. Indeed some *Mycobacterium chelonae* strains isolated from endoscope washer-disinfectors have been shown to be resistant to "in-use" concentrations of GTA, although they remained susceptible to OPA. Although OPA is a more potent microbicide, it is only beginning to become understood as to the reasons why. Studying the interactions of the di-aldehydes at the molecular level, for example with amino-acids, but also at the cellular level, with whole cell micro-organisms, has provided an understanding as to the difference in mycobactericidal activity between the aliphatic and the aromatic di-aldehydes. Notably, an understanding of their reactivity, lipophilicity, cross-linking ability, and penetration mechanism provides an altogether solid explanation for the enhanced efficacy of OPA. Although knowledge of molecular and macro-molecular interactions of a microbicide is important, the study of its interaction with whole cell organisms is necessary to explain overall microbicidal efficacy. This chapter provides an example of such an understanding by reviewing and interpreting the study of di-aldehyde interactions with mycobacteria performed in this laboratory and others.

In New Biocides Development; Zhu, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Introduction

Microbicides (i.e. germicides, biocides) and their use have been described for centuries. Even though early usage concerned mainly the preservation of food and water, in some occasions, transcripts of the use of microbicides to prevent the spread of diseases have emerged, for example, the burning of juniper branch to combat the plague (*1*). The advent of aseptic surgery, as described by Lister, probably marked the onset of their use for medical purposes (*1*). Nowadays, microbicides are heavily used in the healthcare environment for a number of purposes, mainly disinfection, antisepsis, and preservation (of pharmaceutical products) (*2*). Although the same microbicide can be used for different applications, the concentration at which it is used differs. The concentration of a microbicide is of prime importance for its antimicrobial activity (*3*). In addition, some microbicides are more active than others at the same concentration (*4*). Those with a high antimicrobial efficacy are generally more toxic and destined to be used for applications such as surface disinfection, the disinfection of spillages with contaminated materials (e.g. blood, pus, etc.) (*5*), or for the disinfection of heat labile medical equipment, such as endoscopes (*6,7,8,9*). It has to be noted that the use of microbicides for such a purpose, usually referred as high-level disinfection, does not give the same sterility assurance level as physical sterilization processes such as moist heat sterilization (*10*). Nevertheless, the use of microbicides to disinfect medical devices is important and one has to ensure their efficacy (*10,11*). The use of specific standard efficacy tests for such purpose is recommended by several organizations such as the "Comité Européen de Normalisation" (European Committee for Standardization) in Europe (*12*), and the Association of Official Analytical Chemists (AOAC) (*10*) and the Association for Professionals in Infection Control and Epidemiology (APIC) (*13*) in the U.S.A. The stringency of these tests means that only few microbicides can demonstrate sufficient activity to be used for the high-level disinfection of medical instruments. Generally, these can be classified into two main groups, mainly alkylating agents (aldehydes) and oxidizing agents (e.g. hydrogen peroxide, peracetic acid) although others such as chlorine dioxide and superoxidized water have been used (*4, 14*).

Among the aldehydes, glutaraldehyde (GTA) and *ortho*-phthalaldehyde (OPA) are the most widely used for high-level disinfection, on their own or as part of a formulation (*10,14*). It is well acknowledged that several factors can affect the efficacy of microbicides (*15*). Concentration of the active, but also the type of micro-organisms is of particular interest here. Although bacterial endospores are considered to be among the most resistant organisms to disinfection (*15*), mycobacteria represent an interesting challenge. Indeed environmental mycobacterial isolates resistant to "in use" concentrations of GTA have been isolated from endoscope washer-disinfectors (*16*). This chapter

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explores the possible explanation for the difference in mycobactericidal activity between GTA and OPA by looking at their respective mechanisms of action.

Efficacy of Glutaraldehyde and *ortho*-Phthalaldehyde

Efficacy of Aldehydes

The broad-spectrum efficacy of GTA has been well-described in the literature (*17,18,19*). The emergence of microbial resistance to "in use" concentrations (*16*) has been of a particular interest and prompted investigations as to the reason of such failure. The mechanisms of resistance to GTA have not been fully elucidated to date although the high hydrophobicity of the strains might play a role (*20*). Manzoor and colleagues (*21*) showed that an alteration of the cell wall polysaccharides in *Mycobacterium chelonae* contributed towards resistance to the di-aldehyde. For a long time GTA has been a standard microbicide for high-level disinfection and many comparative efficacy studies have used the di-aldehyde. The toxicity of GTA and possibly the emergence of microbial resistance have contributed to the decreased usage of this microbicide. *M. chelonae* subspecies abscessus have been isolated frequently from washer-disinfectors using 2% (v/v) GTA. These strains could cause broncho/endoscope related pseudo-infections in patients (*22*). OPA is a relatively new microbicide which received clearance by the Food and Drug Administration (FDA) in October 1999 for use as a high-level disinfectant (*23*). Several studies have shown the ability of this aromatic di-aldehyde to be used for high-level disinfection (*24,25,26,27,28*). OPA is overall more active than GTA apart from its sporicidal activity (*14*). Most importantly, OPA has been shown to remain active against GTA-resistant *M. chelonae* (*26,27,29*).

Mycobacteria Resistance to Disinfection

Mycobacteria are generally considered intrinsically resistant to microbicides, although they are not the most resistant micro-organisms (*30,31*) (Figure 1). Nevertheless, their innate resistance has been deemed to be caused by their relative impermeability of their cell wall structure (*20*) and notably the presence of a mycolarabinogalactan (Figure 2). Indeed the diffusion of many antimycobacterial agents takes place through the lipid domain of the cell wall, since the hydrophilic pathway is regarded as very inefficient (32). A comparison of GTA-resistant *M. chelonae* with a standard counterpart revealed that the isolates showed an increase overall surface hydrophobicity, but also changes in their cell wall composition, particularly in their arabinogalactan/arabinomanan

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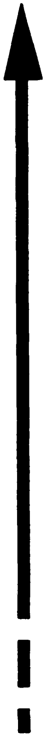

Type of micro-organisms	Comments
- PRIONS	Agents responsible for transmissible spongiform encephalopathy Proteinic in nature; often associated with cell constituents
- BACTERIAL SPORES	Used as biological indicators fro sterilization processes Highly dehydrated; intrinsic resistance also due to their structures (e.g. presence of spore coats)
- PROTOZOAL OOCYSTS	Very little information as to their susceptibility to disinfection. Dehydrated, structure (?)
- MYCOBACTERIA	*M. avium-intracellulare* among the most resistant; environmental *M. chelonae* isolates can show high resistance level (discussed in this chapter)
- SMALL NON-ENVELOPED VIRUSES	e.g. picornaviruses (e.g. poliovirus, echovirus) show some intrinsic resistance to some microbicides
- PROTOZOAL CYSTS	Cysts allow cell survival when challenge to some microbicides
- FUNGAL SPORES?	Very little information available as to the resistance of fungal spores to microbicides – less resistant that bacterial endospores
- VEGETATIVE GRAM-NEGATIVE BACTERIA	Some variation in susceptibility between genera; *Pseudomonas aeruginosa* particularly resistant to some microbicides
- LARGE NON-ENVELOPED VIRUSES	Not as resistant as small non-enveloped viruses. Some variability in susceptibility to microbicides depending upon the type of viruses. Some enteroviruses are particularly insusceptible to some microbicides
- FILMENTOUS FUNGI AND YEASTS	Paucity of information as to their susceptibility to microbicides. Not considered to be a particular challenge
- PROTOZOA	e.g. *Acanthamoeba castelanii*, not considered to be particularly a challenge for microbicides
- VEGETATIVE GRAM-POSITIVE BACTERIA	Generally more susceptible to Gram-negative bacteria
- ENVELOPED VIRUSES	Usually considered to be very susceptible to microbicides (e.g. HIV)

Figure 1. Microbial susceptibility to microbicides. This figure provides an indication of the susceptibility of micro-organisms to microbicides, although variability in susceptibility can be observed between and within types of micro-organisms. The arrow represents the decreasing level of susceptibility to microbicides.

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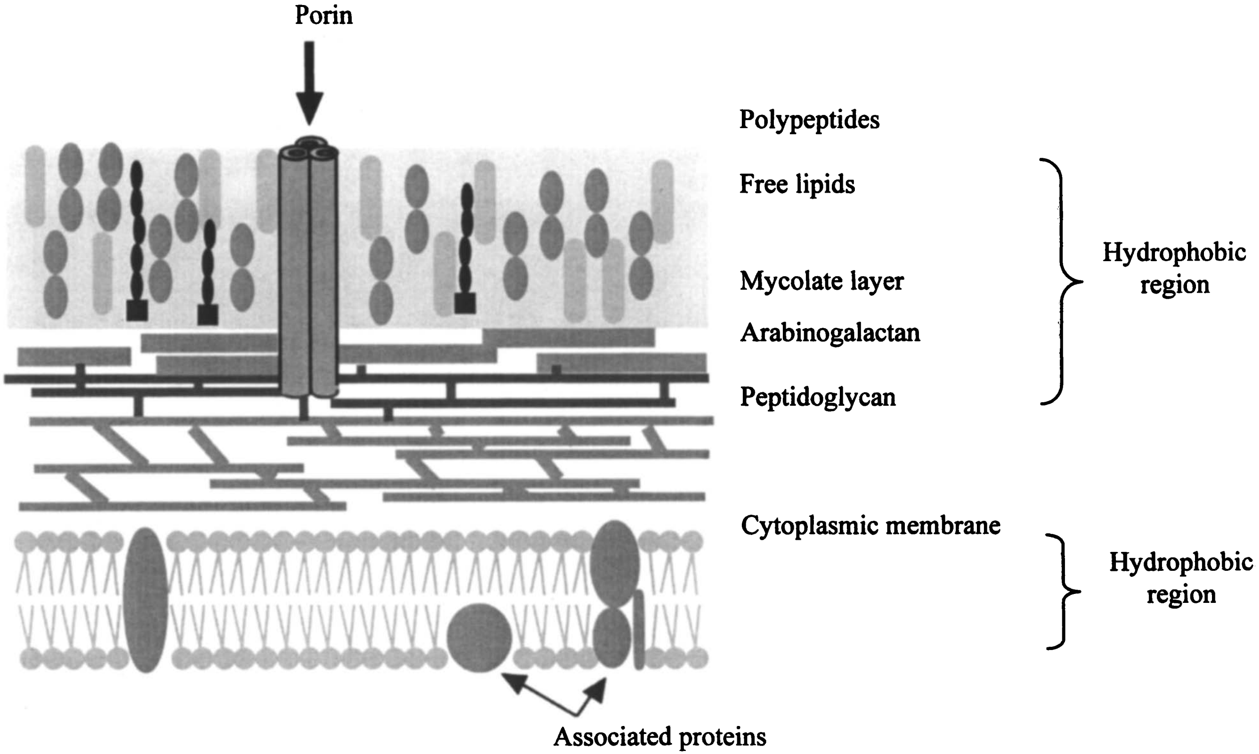
Porin
Polypeptides
Free lipids
Mycolate layer
Arabinogalactan
Peptidoglycan
Hydrophobic region
Cytoplasmic membrane
Hydrophobic region
Associated proteins

Figure 2. Simple schematic of the cell wall structure of mycobacteria

portions (*21*). Many mycobacteria that are slow growing are often more pathogenic. It would be also tempting to assume that such slow growing species would also be less susceptible to antimicrobials than fast growing species. Many studies concerning the efficacy of microbicides against mycobacteria have been based on a planktonic system whereby the challenged micro-organisms are grown in suspension. This might not reflect conditions *in situ,* where micro-organisms are often associated with surfaces and grow as a biofilm. Mycobacterial biofilms have been shown to decrease further the efficacy of GTA and OPA (*33*), although, these results are not unexpected since bacterial biofilms are known to present additional challenge to microbicides (*34*).

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Mycobactericidal Mechanisms of Action of Glutaraldehyde and Ortho-phthalaldehyde

In order to provide an explanation as to the difference in mycobactericidal activity between GTA and OPA, it is necessary to understand better the interactions of the di-aldehydes at the molecular and at the cell levels. Glutaraldehyde (1,5-pentanedial) is an aliphatic di-aldehyde (Figure 3). The effects of pH and temperature on the polymerisation of the molecule and on its lethal activity have been well-reported (*18,19*). At an alkaline pH, GTA molecule undergoes an aldol condensation followed by dehydration to generate α, β-unsaturated aldehyde polymers (*19*). Russell (*18*) pointed out that the availability of the aldehyde group clearly affects antimicrobial activity. OPA is an aromatic lipophilic di-aldehyde (Figure 3) for which activity against vegetative micro-organisms is not pH-dependent. However, an increase in temperature, pH and concentration enhances greatly its sporicidal activity (*26,35*).

Reaction with Amino Groups

GTA has been shown to react strongly with proteins and amino acids (*36*) in various micro-organisms, including mycobacteria (*18*), spores (*26,37,38*), viruses (*39,40,41,42*) and fungi (*43*). GTA reacts with N-terminal amino groups of peptides and α-amino groups of amino acid (*44*). GTA reacts primarily with primary amine and to as lesser extent with secondary amines (*45*). It is accepted that GTA interactions with proteins at pH 8 involves mainly free ε–lysinyl residues (*46,47*). pH has been shown to be more important for GTA reactivity with amino acids (*45*) than for OPA, although increasing pH increases the rate of reaction of the aromatic di-aldehyde (*49*). In addition, GTA does not interact with histine whereas OPA does (*48*). OPA also reacts strongly with amino acids,

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particularly with amino group (*49*) rather than thiol groups for which reaction is much slower. However, its reactivity can be increased by the addition of an excess of thiol groups (*50,51*). Such a property has been used for the detection of amine-containing molecules via the formation of fluorogenic isoindoles between OPA and a thiol compound (*52,53*). OPA reacts readily with primary, secondary (histidine and tryptophan) and quaternary ammonium ion (NH_4^+)(*49,54*). The activity of aldehyde-based microbicides is linked by their interactions with amino groups (*45,55*), notably to their ability to react and cross-link (see below) with primary amines. Indeed in the microbial cell, amino acids (containing primary amines) are abundant within their structure (e.g. cell wall) but also among various enzymes.

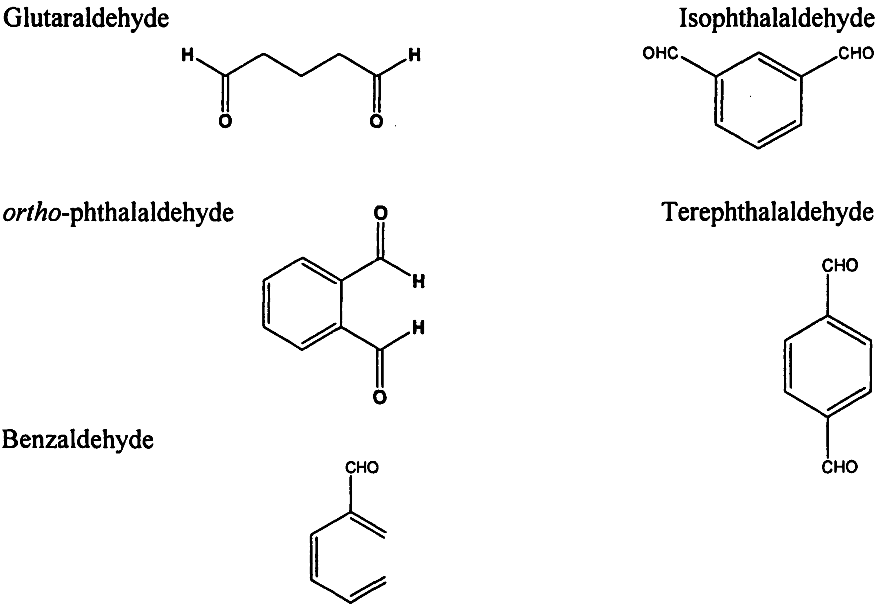

Figure 3. Structure of some aldehydes

Cross-Linking Ability

Exposure of proteins to GTA has been shown to produce products that are not dissociated by dilution and that are resistant to hydrolysis (*19,56*). Indeed several studies with different micro-organisms have shown that pre-treatment with GTA of bacterial spheroplasts prevented the lysis of the cell when exposed

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to water or lytic agents (*49,57,58,59,60,61*). It was shown that GTA reacts and cross-links primary amine groups of cell components (*18,19,62,63,64*). GTA polymer acts as an electrophile under physiological conditions and as such can cross-link effectively with nucleophile (i.e. amino group) in a Michael type 1,4-addition reaction similarly to other α,β-unsaturated aldehydes (*65,66,67,68*). Pre-treatment of bacterial spheroplasts with OPA resulted to their protection from hydrolysis or lysis from various chemicals, but not to the same extent as GTA (*49,69*). Interestingly, Walsh et al. (*49*) observed that rising pH made little difference in the cross-linking conferred-protection by GTA or OPA from EDTA-induced lyses in *Pseudomonas aeruginosa*. GTA increased efficacy at alkaline pH is likely to be a combination of enhanced cross-linking ability, but also changes to the cell surface (*17*). Both GTA and OPA were able to prevent the lysis of *Staphylococcus aureus* exposed to lysostaphin (*49*). Russell and Vernon (*60*) postulated that GTA interaction with *S. aureus* cell reduced or prevented the action of the lytic peptidase principle. It would be tempting to assume that OPA interacts similarly to GTA with *S. aureus*. Fraud and colleagues (*69*) reported that GTA pre-treated mycobacterial spheroplasts were less affected by osmotic shock-induced lysis than OPA pre-exposed ones.

Simons and colleagues (*48*) postulated that the apparent decreased cross-linking ability of OPA was caused by the decreased reactivity in nucleophilic addition reactions of aromatic aldehydes when compared to aliphatic ones, but more importantly the steric restrictions of the planar and rigid benzene ring of OPA. Addressing the physical requirement for cross-linking abilities of both GTA and OPA can provide further information (*48,69*). Hughes and Thurman (*70*) calculated that the distance between contiguous peptides along a single polysaccharide backbone would be less than 10.3 Å. GTA should be able to cross-bridge this distance (*47*) and effectively cross-link, whereas OPA would only be able to bridge effectively a <3 Å distance (71). However, such an explanation does not provide an appropriate answer as to why pre-exposure to OPA protects bacterial spheroplasts to a greater extent than mycobacterial ones. Fraud *et al.* (*69*) pointed out that bacterial spheroplasts contained more structures containing side-chains. This would provide some explanation as to the difference in conferred-lysis protection following OPA pre-treatment between mycobacterial and bacterial spheroplasts.

The importance of peptidoglycan as a target site for aldehyde interactions (*69*) was highlighted in a study providing some explanation as to the development of GTA-resistance in *M. chelonae* (*21*).

More importantly, the difference in cross-linking ability between GTA and OPA provides an explanation as to the reason why GTA is generally slower at inactivating micro-organisms (*69*). Indeed the extensive cross-linking ability of GTA would decrease its own uptake, whereas OPA might penetrate faster within the cell.

Lipophilicity of Aldehydes

In mycobacteria, the hydrophilic pathway is highly inefficient (*32*). Lipophilic molecules are thus expected to penetrate the cell more readily. If this is the case, the lipophilic nature of OPA (log *P*oct = +1.53)(*72*), would explain its increased efficacy when compared to GTA. An interesting study compared the activity of several aldehydes against various mycobacterial isolates (*27*). Results extracted from this study highlighting mycobactericidal activity in relation to aldehyde lipophilicity (Table 1) showed a clear trend for lipophilic aldehydes to be more active. On exception, the aliphatic glyoxal was not mycobactericidal at a concentration of 0.5%, although it was observed to be more active at 10% (*27*). The lipophilic mono-aldehyde benzaldehyde was highly mycobactericidal (Table 1), which adds evidence to the importance of lipophilicity and argument the increased penetration potential of the aldehyde through the outer layers of mycobacteria. However, this aromatic mono-aldehyde was not active against the glutaraldehyde-resistant *M. chelonae* Harefield and Epping (Table 1). In addition another two aromatic di-aldehydes, structural analogues of OPA with a similar lipophilicity, isophthalaldehyde and terephthalaldehyde (Figure 3) were found to be inactive against *Mycobacterium bovis* unlike OPA (*73*). This provides evidence that although lipophilicity is important other factors must be taken into account to explain the mycobactericidal activity of OPA.

Penetration of Aldehydes

It is clear that lipophilicity on its own can not explain the increased efficacy of some di-aldehydes. The study of the structural analogues of OPA, isophthalaldehyde and terephthalaldehyde, has led Zhu et. al. to propose a specific mechanism for OPA penetration, which involves a "molecular switch" when the molecule is hydrated (*73*). It was proposed that the hydrophobe ("opened" form) of OPA and the hydrophile 1,3-phthalandiol ("locked" form) coexists depending upon the medium. A higher proportion of the "locked" form (approx. 90%) is present when hydrated (*74*). Based on these findings, the authors (*74*) proposed a "medium-induced molecular switching" model to explain the enhanced penetration of OPA through the bacterial cell wall. While in the 1,3-phthalandiol form, the molecule is unable to interact with proteins and lipids. However, when going through the hydrophobic region of the cell (e.g. the lipid reach outer layers and the cytoplasmic membrane in mycobacteria (Figure 2)), the "locked" form switches to the "open" form (OPA) which then predominates. By switching between the hydrophobe and the hydrophile forms while going through the cell layers, OPA should be able to penetrate deeper within the cell (*74*). The cell cytoplasmic membrane most probably represents

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Table 1. Mycobactericidal activity of aldehyde vs. lipophilicity

Aldehydes	Log P	Concentration (%)	*M. chelonae*	*M. chelonae Epping*	*M. chelonae Harefield*	*M. abscessus*	*M. terrae*	*M. Smegmatis*
Succinaldehyde	-1.17	0.5	0*	0	0	0	0	NT
Malonaldehyde	-1.11	0.5	0	0	0	0	0	NT
Glutaraldehyde	-0.75	0.5	>5 (2-5 min)	0	0	>5 (30-60 min)	>5 (5-10 min)	>5 (1 min)
Glyoxal	-0.37	0.5	0	0	0	0	0	NT
ortho-phthalaldehyde	+1.53	0.5	>5 (1 min)	>5 (1 min)	>5 (1 min)	>5 (5-10 min)	>5 (1 min)	>5 (1 min)
Benzaldehyde	+1.78	0.396	>5 (1 min)	0	0	>5 (1 min)	0	>5 (1 min)

*Log_{10} reduction in viable colony obtained (within min); 0: no reduction; NT: not tested

Based on Fraud and Colleagues (*27*)

the most important target site, where enzymes responsible for cell respiration, transport, synthesis of cell wall constituents, general metabolism, etc. might be present. Thus the ability of OPA to reach and interact with the cytoplasmic membrane as an "opened" form would explain its increased efficacy. In addition, such a model explains the lack of activity of isophthalaldehyde and terephthalaldehyde which are unable to form such a "locked" configuration. Although this concept is interesting, it might not provide an explanation as to the efficacy of aromatic mono-aldehydes such as benzaldehyde (Table 1).

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Enhanced Mycobactericidal Action

To explain the increased mycobactericidal efficacy of OPA when compared to GTA, factors related to the molecule, such as carbon length and degree of bond saturation (*75,76*), and its reactivity with amino and thiol groups have to be taken into consideration, together with factors that affect the penetrability of the molecule such as lipophilicity and cross-linking ability. For aliphatic aldehydes, such as GTA, the presence of α,β-double bonds but also the chain length of the enal group is important (Table 1). In addition, the propensity of GTA to irreversibly bind to and extensively cross-link its targets, mainly by stable Michael addition type reactions (*65*), and the availability of multiple targets, would explain its lack of penetration through the mycobacterial cell envelope and its effective but slow mechanisms of mycobactericidal action.

The mycobacterial cell envelope offers multiple target sites to the aldehydes (Table 2; Figure 2) from the outer cell layer to the cytoplasmic membrane. Although the composition of the cell envelope varies with the species and possibly the strains (e.g. glutaraldehyde-resistant *M. chelonae*) (*21*), the number of targets for the aldehyde is likely to remain high, increasing cross-linking and the subsequent depletion of the aliphatic GTA. Indeed there is no evidence that GTA penetrates the cytoplasm of the cell and there are questions as to what extent it reaches the cytoplasmic membrane.

On the other hand, the aromatic OPA is likely to penetrate through the mycobacterial cell envelope and there is some evidence that it can reach the cell cytoplasm, with the observed coagulation of *M. chelonae* spheroplasts (*69*). The combination of higher lipophilicity, reduced cross-linking ability and a medium-induced molecular switch mechanism play an essential part in the enhanced penetration of the aldehyde through the mycobacterial cell envelope. Allowing a high concentration of OPA to penetrate deeply, presumably reaching the cytoplasmic membrane and cell cytoplasm, provides a rationale for its increased mycobactericidal efficacy.

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Table 2. Composition of the mycobacterial cell wall

Cross-section	Composition (possible constituents)	References
Outside layer (surface)	Free lipids, glycopeptidolipids, trehalose-containing lipooligosaccharides, sulpholipids, phthiocerol dimycocerosate, phenolic glycolipids phosphatidyl-ethanolamine, high concentration of proteins and polysaccharides, lipoarabinomannan, mycolic acid, arabinan, galactan	(*77,78,79,80*)
Cell wall	Peptidoglycan, sulfhydryl-containing lipids, peptide and porin-like proteins	(*78*)
Periplasmic space and cytoplasmic membrane	Numerous proteins (inc.enzymes and porins), phospholipids (phosphatidylinositol mannosides, phosphatidylinositol mannosides, phosphatidylglycerol, cardiolipin (diphosphatidyl-glycerol) and phosphatidylethanolamine; phosphatidylinositol.	(*77,78*)

Conclusion

Microbicides – Interactions with Macromolecular Targets

Knowledge of microbicide interactions with macromolecular targets, such as proteins and nucleic acids, is important and enables one to predict to some extent their lethal or inhibitory efficacy (*81*). Reactivity with specific groups such as amino and thiol adds to the understanding as to whether or not a microbicide has the propensity to be highly reactive. Such information also provides an understanding as to the amount of the agent that will be depleted when used against a target micro-organism; i.e. the concentration of a highly reactive agent is likely to decrease more rapidly following interaction with a high number of target sites. This is indeed observed with the interaction of GTA with the mycobacterial cell. Such an understanding forms the basis of structure-activity relationship analysis.

One possible drawback is that knowledge of the interaction between a microbicide and a specific target site such as an enzyme can lead to some degree of confusion as to its activity and subsequent application in practice. For example, the bis-phenol triclosan has been extensively investigated and found to inhibit specifically the enoyl-acyl carrier protein reductase in several micro-organisms (*82,83,84,85*). Triclosan acts as a potent irreversible inhibitor (*86,87*). Such specificity questioned the usefulness of the microbicide notably in light of the emergence of microbial resistance through modification of the enzyme (*88*) However, it is now clear that the lethal efficacy of the bis-phenol is not solely caused by the inhibition of the enoyl reductase, but by an interaction with multiple target sites (*89,90*).

Nature of Biocidal Agents – Multiple Target Sites and Resistance Mechanisms

A microbicide by definition has multiple target sites within the bacterial cells, and the level of interactions and the amount of damage resulting from such interactions produce an inhibitory or lethal effect (*81*). Therefore, the investigation of the mechanisms of action of a microbicide should include its interaction with the whole cell. This is particularly relevant to highly reactive compounds such as alkylating agents (e.g. aldehydes) and oxidizing agents (e.g. peroxygens). Other microbicides such as quaternary ammonium compounds, phenolics and biguanides have a more selective interaction with the cell, even though they are likely to interact with multiple target sites notably at the cell membrane level (*4,30*).

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To explain the "lethal" efficacy of a microbicide by its mechanisms of interactions with the microbial cell is further complicated by the intrinsic nature or the response of the micro-organisms to the microbicide challenge. Different classes of micro-organisms have different susceptibility to microbicides, often as a result of an intrinsic property (Figure 1) (*15,30*). As such, the study of clinical isolates often provides some interesting findings. For example, the endoscope-washer isolates *M. chelonae* Harefield and Epping have been shown to be resistant to glutaraldehyde (*16,26*) and other aldehydes (*27*), but not to OPA (*21*). While a modification in cell wall polysaccharide might explain GTA-resistance (*21*), it is unclear as to why OPA remains active. Although the increase penetration of the microbicide is likely to play a role, an increased lipophilicity can not explain solely such activity. Studying the mechanisms involved in the intrinsic or acquired resistance of micro-organisms should be viewed as part of the understanding of microbicide-cell interactions. To this effect novel methodology such as the use of transposon mutagenesis might prove very useful (*91*).

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OPA *vs.* GTA

The study of the interactions between a microbicide and a specific micro-organism can provide an understanding as to its lethal efficacy. To this effect this chapter has provided some explanations as to the enhanced activity of the aromatic OPA *vs.* the aliphatic GTA against mycobacteria. The difference in mycobactericidal activity can be explained by a differential in penetration and reactivity with the cell outer layers. GTA interacts strongly with the cell upper hydrophobic region, resulting in extensive cross-links with proteins and lipids preventing the di-aldehyde from penetrating deeper within the cell and it remains unclear as to whether or not GTA reaches the cytoplasmic membrane. On the other hand, the lipophilicity of OPA, its reduced cross-linking ability, and a possible molecular switching mechanism enable the aromatic di-aldehyde to reach targets deep within the cell structure, presumably the cytoplasmic membrane and even the cytoplasm. Such interactions explain the enhanced mycobactericidal activity of OPA and might also provide an explanation as to its efficacy against GTA-resistant mycobacteria.

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Chapter 9

ortho-Phthalaldehyde: Mechanisms of Action against Mycobacteria

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In recent years, *ortho*-phthalaldehyde (OPA) solutions have emerged as alternatives to glutaraldehyde solutions for high-level disinfection of semicritical medical devices. The increased use of OPA-based disinfection solutions is due in part to the antimicrobial activity of OPA against glutaraldehyde-resistant mycobacteria. In this chapter we review the available information on the mechanisms of action of OPA against mycobacteria, which are well known for their resistance to many different chemical germicides. The unique cell wall architecture of the mycobacteria, measurements of cell surface hydrophobicity, and resistance to aldehyde-based disinfectants are discussed.

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Disinfection can be defined as a chemical process that is capable of destroying most types of disease-producing microorganisms on inorganic surfaces. Disinfectants are commonly divided into different classes based on their effectiveness against different microorganisms. Three disinfectant categories are typically recognized: "low-level," which kills most vegetative bacteria and some viruses and fungi, but not mycobacteria or bacterial spores; "Intermediate-level," which kills vegetative bacteria, most viruses and all fungi, but does not kill spores; and "high-level," which kills all microorganisms except high numbers of spores.

A classification system for medical instrumentation devised in the late 1960's by Earle Spaulding is commonly used to determine the appropriate methods for preparation of medical instruments prior to use. The clinical utilization of the instrument determines the level of disinfection required. The three categories of instruments or devices are: "critical," introduced into normally sterile areas of the body or into the vascular system; "semicritical," which contact intact mucous membranes and usually do not penetrate body surfaces; and "noncritical," which contact only intact skin. High-level disinfection is the minimum treatment recommended for the reprocessing of semicritical medical instruments.

Glutaraldehyde-based high-level disinfectants (HLDs) were first introduced for this purpose in 1963 and have been the disinfectants most often used for high-level disinfection since that time (*1*). In the 1970's, reports surfaced of mycobacteria that exhibit increased resistance to these glutaraldehyde-based disinfectants.

In response to the threat posed by glutaraldehyde-resistant mycobacteria, *ortho*-phthalaldehyde solutions (OPA) have emerged as an alternative to glutaraldehyde solutions for high-level disinfection of reusable medical devices, especially flexible endoscopes (*2*).

Bruckner et al. disclosed in a 1990 patent (*3*) a sterilizing and disinfecting solution containing a saturated dialdehyde and an aromatic dialdehyde. The aromatic dialdehyde contributed to a more rapid kill of *Mycobacterium bovis* (BCG) at 20° C without diminishing the capability of the solution to destroy other microorganisms, including bacterial spores. The preferred aromatic dialdehyde was found to be phthalaldehyde due to its reasonable solubility in water and tuberculocidal activity.

Examples showed that when several aromatic dialdehydes, including phthalaldehyde, were added to 2% glutaraldehyde solutions, the solutions demonstrated enhanced tuberculocidal activity. A separate example showed that phthalaldehyde had excellent tuberculocidal activity by itself. Also demonstrated was that with as low as 0.005% (w/w) phthalaldehyde, solutions of 2% glutaraldehyde are tuberculocidal within 30 minutes at 20° C. Similarly, solutions containing either 0.75% glutaraldehyde and 0.01% phthalaldehyde or

0.25% glutaraldehyde and 0.025% phthalaldehyde also are tuberculocidal within 30 minutes at 20° C. The tuberculocidal activity was not pH dependent (pH 3-9). The addition of 0.1% phthalaldehyde did not inhibit sporicidal activity of 2% glutaraldehyde.

Chemical Properties

In contrast with the aliphatic glutaraldehyde (GTA), *ortho*-phthalaldehyde is an aromatic dialdehyde compound with the formula $C_6H_4(CHO)_2$ and a chemical (IUPAC) name of 1,2-benzenedicarboxaldehyde with the following structure (Figure 1):

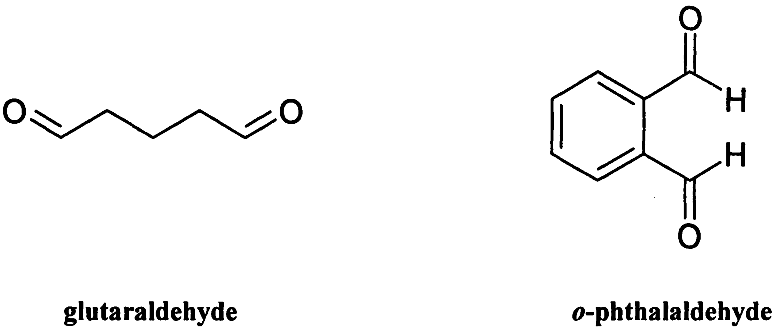

Figure 1. Comparison of glutaraldehyde and ortho-phthalaldehyde (1,2-benzenedicarboxaldehyde) chemical structures.

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A commercially available OPA-based disinfectant is marketed as CIDEX®OPA Solution and contains 0.55% w/v *ortho*-phthalaldehyde and was introduced into the market in 1999. Like glutaraldehyde, OPA is a dialdehyde. However, OPA is an aromatic compound, whereas glutaraldehyde is a straight chain hydrocarbon.

Mycobacteria

Mycobacteria as a genus include many pathogens known to cause serious diseases in humans and animals, including tuberculosis and leprosy. Mycobacteria are generally classified into two categories based on growth rate: fast-growing species such as *M. chelonae* and *M. smegmatis* and slow-growing species like *M. tuberculosis* and *M. avium intercelluare.* Tuberculosis (TB) is the world's leading cause of death that results from a single infections disease. TB affects an estimated 1.7 billion people, equal to almost one-third of the entire global population.

Mycobacteria are widespread in the natural environment, typically living in water (including tap water treated with chlorine), food sources and decaying organic material. As a genus, they share characteristic cell walls that are thicker than those of many other bacteria and which are hydrophobic, waxy and rich in mycolic acids / mycolates. The mycobacterial cell wall is responsible in large part for the hardiness of this genus. Mycobacterial infections are notoriously difficult to treat and because of this unique cell wall can survive long exposure to acids, alkalis, and detergents. Since mycobacteria represent the most difficult to kill vegetative microorganisms, they are commonly used to measure the efficacy of disinfectants.

The Action of *o*-Phthalaldehyde on Mycobacteria

In contrast to chemotherapeutic agents, biocides have multiple targets within the microbial cell, and the overall damage to target sites results in the biocidal effect (*4*). *ortho*-Phthalaldehyde's primary mechanism for biocidal activity is the reaction with primary amino groups (Figure 2) found within multiple bacterial structures, and in particular with proteins of the cell wall (*5*).

To reach proteins at the cell wall, antibacterial agents such as OPA must first diffuse through the mycobacteria's thick, lipid-rich protective coat, comprised of mycolic acids. Mycolic acids are long chain, beta-hydroxy-alpha-

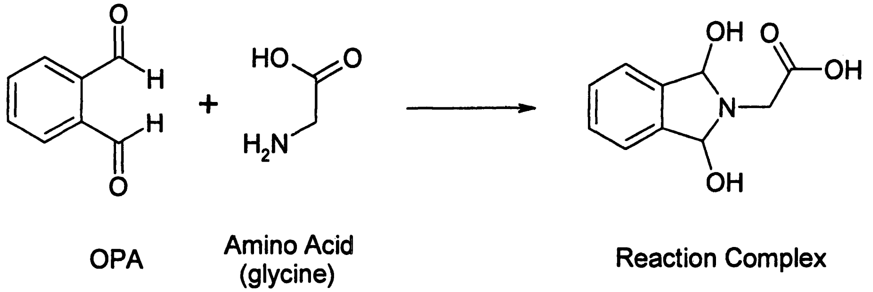

Figure 2. Reaction of OPA with a primary amino acid (glycine), the primary mechanism by which OPA denatures critical mycobacteria cell wall proteins.

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alkyl branched fatty acids and in mycobacteria are covalently linked to an arabinogalactan polymer. The bacterial cytoplasmic membrane is further protected by a peptidoglycan matrix linked to the arabinogalactan layer. This defensive structure featuring long chain fatty acids serves as a protective outer wall for the mycobacteria cells and can effectively exclude many types of biocides and antibacterial agents. However, lipophilic agents may utilize the lipid bilayer pathway to cross the cell wall, and this knowledge has driven the rational design of anti-tuberculosis drugs (*6*). In fact, "the lipophilic nature of the molecule will play a key role in the diffusion through hydrophobic domains such as biological membranes."(*7*) As such, the high lipophilicity of OPA is likely the predominant reason why the molecule exhibits much higher efficacy than other dialdehydes.

To demonstrate the relationship between the lipophilicity of biocides and their efficacy against mycobacteria, an examination of cell surface hydrophobicity was performed using two strains of mycobacteria. In such an experiment, an aqueous suspension of *M. chelonae* was combined with an organic layer (hexadecane). After mixing and settling, the relative populations of the *M. chelonae* in each layer were examined.

The degree to which cells were found to populate the organic layer as compared with the numbers remaining in the aqueous layer can provide some insight into their relative cell surface hydrophobicities (Figure 3). Mycobacteria cells that have a more hydrophobic outer coating should be found to a greater extent in the organic phase, as compared with a more hydrophilic species which would tend to remain in the aqueous phase. Two strains were chosen for testing: *M. chelonae* ATCC 35752 was chosen to represent the "typical" mycobacteria, for comparison against a glutaraldehyde resistant strain of *M. chelonae*. Interestingly, the GTA resistant strain of *M. chelonae* partitioned into the organic phase to an extent nearly three times greater than that of the less resistant strain (Figure 4), which clearly indicates a correlation between biocide resistance and the hydrophobicity of the cell wall.

In a companion study, the mass fraction of a primary source of the cell surface hydrophobicity, fatty acid methyl esters, was compared for the two strains. The data (Table 1), indicated nearly three times the tuberculostearic acid in the GTA-resistant mycobacteria, a result consistent with the results of the partitioning experiment. However, a causative correlation is not entirely clear.

Clearly the mechanism of glutaraldehyde resistance in GTA-resistant mycobacteria is related to its significantly higher cell surface hydrophobicity. The higher hydrophobicity tends to exclude molecules which are polar in nature, and to some extent also excludes glutaraldehyde, which has an octanol-water partition coefficient (K_{oc}) of only 16. *o*-Phthalaldehyde, however, is a considerably less polar molecule ($K_{oc} = 45$), which is much more soluble in nonpolar phases (*8*). This solubility lends OPA its particular efficacy against

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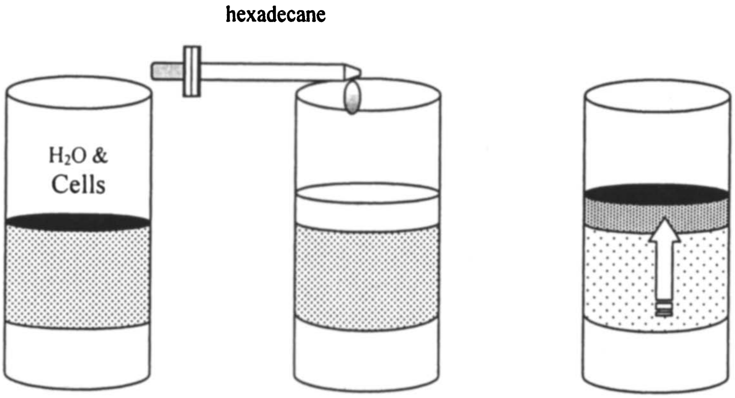

Figure 3. Schematic diagram of cells originally found only in the aqueous phase partitioning between aqueous and organic phases. Cell Hydrophobicity Assay relates the probability of finding cells in the aqueous or organic phase to the cell surface hydrophobicity.

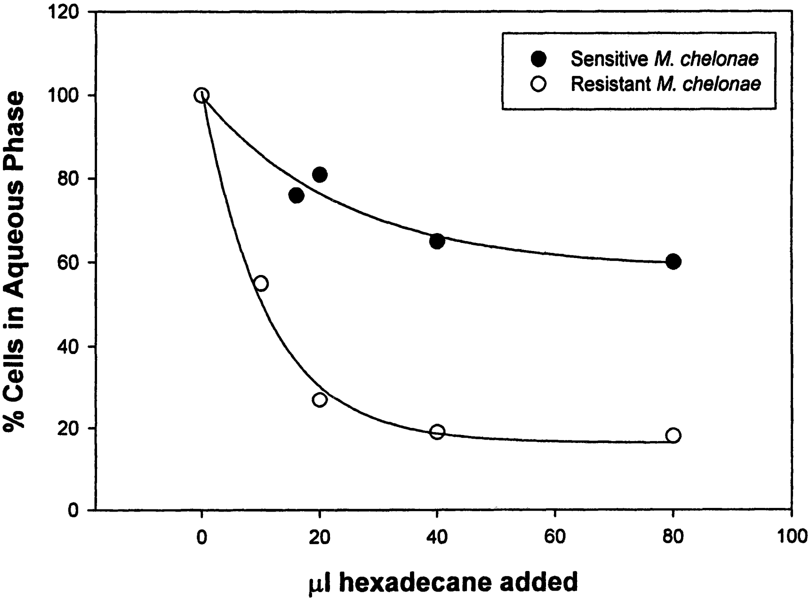

Figure 4. The relationship between cell hydrophobicity and glutaraldehyde resistance was demonstrated using a Cell Hydrophobicity Assay. GTA resistant M. chelonae partition into the organic phase to a far greater extent than sensitive M. chelonae which mostly remained in the aqueous phase.

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Table I. Glutaraldehyde Resistant Mycobacteria: Cell Wall Fatty Acids

	Mass Fraction FAME	
Fatty Acid Methyl Ester (FAME)	*M. chelonae* (ATCC 35752)	*M. chleonae* (GTA Resistant)
n-hexadecanoate (palmitate)	7.9	19.1
D-10-methylstearic acid (tuberculostearic acid)	5.5	15.6
cis-9-octadecanoate (oleate)	36.9	29.9

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mycobacteria as its compact, aromatic structure allows it to traverse the lipid layer that might exclude more polar compounds.

In a subsequent experiment, GTA-resistant *M. chelonae* was exposed to both OPA and glutaraldehyde for a comparison of their relative efficacies. The data (Figure 5) clearly indicated that OPA's lipophilicity allowed for enhanced permeation across the mycobacteria's lipid layer, resulting in a total kill (6-log reduction) of bacteria in less than 15 minutes. Equivalent samples of mycobacteria treated with glutaraldehyde exhibited more than 100 survivors, even after 20 minutes.

When compared with several other biocide molecules with lower lipophilicity including GTA, glyoxal, glyoxal and butanedial, 5-min kill data for *M. abscessus* from Fraud et al. (*9*) indicated the clear superiority of OPA. Interestingly, when the calculated octanol water partition coefficients were compared with efficacy, a generally linear correlation was observed between the biocide efficacy and the ability to diffuse through hydrophobic domains.

It should be noted that while lipid membranes are highly permeable to lipophilic molecules such as OPA, several additional factors can inhibit overall permeability of cell walls. For example, membrane fluidity decreases as the hydrocarbon chains lengthen and contain fewer *cis*-double bonds and *cis*-cyclopropane groups. Also, drug resistant strains of *M. chelonae*, *M. fortuitum*, *M. smegmatis* and *M. phlei* contain a larger fraction of α-mycolates with less permeable *trans* double bonds at the inner position (*6*). Glutaraldehyde-resistant *M. chelonae* was found to not only exhibit increased cell surface hydrophobicity as above (*10,11*) but they also exhibited decreases in the monosaccharides of arabinogalactan (*11*). Finally, the protein cross-linking action of glutaraldehyde

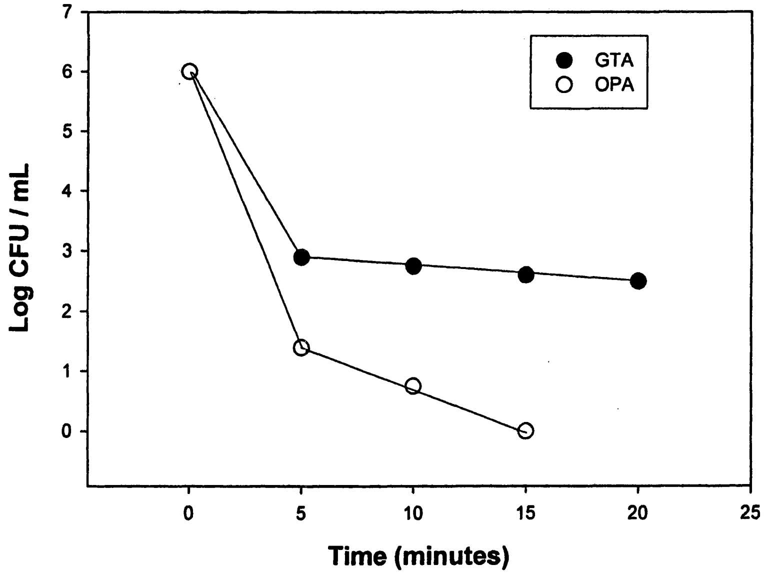

Figure 5. Comparison of relative activities of OPA and GTA for the inactivation of a GTA resistant strain of M. chelonae.

may ultimately inhibit its own uptake, whereas OPA is not an effective cross-linking agent, presumably due to the spatial relationship of its aldehyde groups.

Molecular Simulations of the Action of *o*-phthalaldehyde

While it is difficult to experimentally determine the individual contributions of molecular structure and lipophilicity to the overall efficacy of a biocide against mycobacteria, molecular dynamics simulations can provide some insight.

In cooperation with Anton J. Hopfinger of the College of Pharmacy at the University of New Mexico, computer simulated mycobacterial lipid layers were challenged with models of OPA and glutaraldehyde. MOLSIM software was used to generate a total of 140,000 confirmations of the biocides in 0.001-ps intervals to determine overall permeability of the layer for each of the molecules.

The results of molecular dynamics simulations (Figure 6) suggest that the enhanced efficacy of OPA over glutaraldehyde against mycobacteria could be almost entirely derived from the improved permeability afforded by OPA's lipophilic structure. The data suggested that given similar reactivities, biocides

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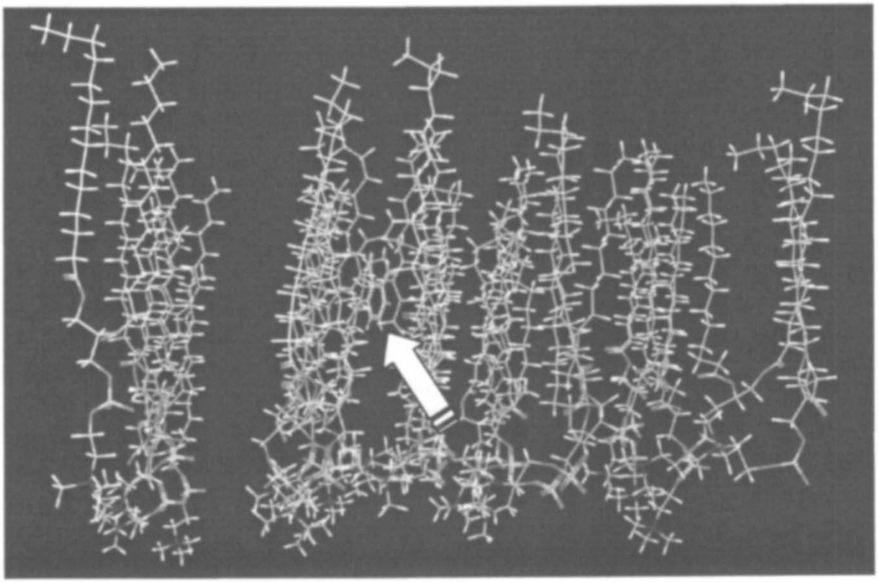

Figure 6. MOLSIM screen shot, detailing the progression of unhydrated OPA through a simulated layer of mycolic acids.

that are compact and highly lipophilic will exhibit the greatest efficacy against mycobacteria, information useful in the rational design of biocide molecules.

The Equilibria of *o*-phthalaldehyde

An additional complexity of OPA is the molecule's ability to undergo covalent hydration in an aqueous environment. In fact, because of the electron-withdrawing and "*ortho*-effect" of the second carbonyl group, the equilibrium is shifted toward the hydrated form. As the electron-withdrawing properties of the $CH(OH)_2$ group are not as substantial as those of a CHO group, the addition of a second water molecule is not favored. Instead, an intramolecular nucleophilic attack of an oxygen atom of the $CH(OH)_2$ group on the remaining carbonyl group occurs, resulting in a cyclic hemiacetal (*12*) (Figure 7). These equilibria potentially play a role in the inactivation of mycobacteria with OPA, as the hydration of OPA results in structures with octanol-water partition coefficients resembling that of glutaraldehyde. While in principal hydrated forms of OPA may react with nucleophiles, their ability to cross the mycobacteria lipid layer may be limited by their increased polarity.

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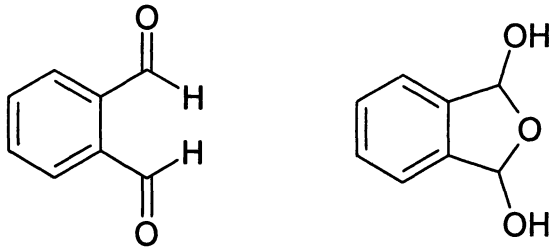

Unhydrated OPA Hydrated OPA

Figure 7. Comparison of the unhydrated OPA structure with the hydrated cyclic hemiacetal. In an aqueous biocide solution, about 10% exists in the unhydrated form while about 70% reacts with water to form the cyclic hemiacetal (13). About 20% exists as an acyclic monohydrate (not shown).

Conclusions

It is generally thought that the primary action of *o*-phthalaldehyde on mycobacteria is the denaturing of critical proteins through the reaction with primary amino groups. The rate limiting step in the inactivation of mycobacteria appears to be the diffusion of biocides through the bacteria's waxy, outer coating. Biocides that have the highest lipophilicity appear to have the greatest efficacy, which is apparently related to their ability to permeate though mycolic acids and other hydrophobic regions of the outer cell wall. Glutaraldehyde resistant mycobacteria exhibit considerably higher cell surface hydrophobicity than other mycobacteria, which suggests that their outer structures effectively exclude more polar compounds such as GTA from sensitive, inner structures. The enhanced efficacy of OPA for the inactivation of glutaraldehyde resistant mycobacteria can be explained almost entirely by its significantly higher lipophilicity.

Once biocide molecules such as OPA have traversed the waxy outer layers, from a strictly probabilistic viewpoint they will attack amino groups in the sequence as encountered by diffusion. Those amino groups found within the arabinogalactan-peptidoglycan complex, therefore, would likely be primary targets leading to the inactivation of the cell. Proteins of the cytoplasmic membrane would also be vulnerable to attack. While it is likely that OPA has intra-cellular targets, it is entirely possible that inactivation needs only to result from sufficiently denatured arabinogalactan, peptidoglycan and cytoplasmic membrane layers. OPA molecules which diffuse through the many cell wall

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layers and enter the cytoplasm may attack intracellular targets. Though due to the lack of DNA mutagenicity, it is likely that the principal reactive sites for OPA lie within the arabinogalactan-peptidoglycan complex and cytoplasmic membrane layer. Cell penetration may not be necessary for efficacy.

The predominant OPA species in an aqueous solution of OPA biocide is the hydrated cyclic hemiacetal. However, it is likely based on octanol water partition coefficients (K_{OC}) and molecular dynamics simulations that the unhydrated OPA species will have the greatest propensity to permeate through mycobacteria's protective outer lipid layer. Hydrated OPA, glutaraldehyde, and other less lipophilic biocide molecules found on the outside of the cell wall will be less likely to permeate into the lipid layer. In effect, it is likely that 10% of an OPA biocide solution provides most of the mycobactericidal efficacy.

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Chapter 10

The Jekyll and Hyde Roles of Cysteine Derivatives during Oxidative Stress

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Cysteine is one of the least frequently employed amino acids in biosynthesis (*1*), presumably due in part to its reactivity relative to other amino acids, particularly with respect to oxidation. However, it is this reactivity that is exploited by many defensive mechanisms for oxidative stress. Nonetheless, the reactivity of the sulfhydryl group can be burdensome when reactive sulfur species (*2-4*) are formed. Ironically, there is mounting evidence that under certain circumstances, derivatives of cysteine can act as pro-oxidants. We focus our attention in this report on the apparent deleterious roles of cysteine in the presence of the archetypal biocides hydrogen peroxide and hypochlorite.

Introduction

Although biocides have been used for centuries, there is still a dearth of information regarding their antimicrobial mechanisms. Information is also sparse regarding the defenses that are staged by the target organisms. Biocides can be divided into oxidizing (e.g., chlorine/hypochlorite, chlorine dioxide, chloroisocyanurates, ozone, and hydrogen peroxide) and non-oxidizing (e.g.,

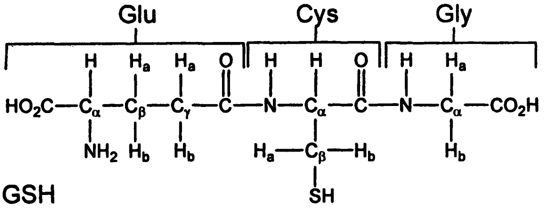
In New Biocides Development; Zhu, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

acrolein, amines, chlorinated phenolics, copper salts, and quaternary ammonium salts) varieties that probably operate via distinctive mechanisms (*5*). Nonetheless, it is generally accepted that all biocides target multiple sites within the microbial cell and that it is the resulting overwhelming damage to these target sites that produces bactericidal effect. Biocides affect multiple biological functions. In contrast, antibiotics, and other chemotherapeutics, frequently target specific steps in biochemical pathways. The application of biocides is not always cytocidal. Bacteriostatic effects are often observed at lower concentrations of a biocide, where life functions may be impaired but not overwhelmed. Furthermore, in an unnerving parallel to the development of antibiotic-resistant microbial flora, resistance to biocides has also been reported. In fact, it has been suggested that biocide and antibiotic resistance in bacteria might be linked (*6*). Thus, a better understanding of the mechanisms of the action of biocides has become a critical issue.

This contribution focuses on two archetypal biocides, hydrogen peroxide and hypochlorite (bleach). Particular attention is placed here on discussing the reactions of these biocides with cysteine (and its derivatives). The title is a reference to a novel by Robert Louis Stevenson that explores the duality of man's nature (*7*). In addition to describing the well-understood beneficial role of thiols as antioxidants, we will describe on-going studies that are aimed at elucidating the possible deleterious effects of secondary reactive species that are formed from biological systems that contain biocidal oxidants and thiols.

Role of Cysteine Derivatives as Antioxidants

Thiols are particularly susceptible to oxidation, so it is not surprising that they are frequently employed as intracellular reducing agents to protect biological systems from oxidative stress. For example, the small tripeptide glutathione (GSH) is found in millimolar concentrations in the cytosols and mitochondria of eukaryotes (e.g., 2 mM in erythrocytes) (*8*). The interesting issue of why glutathione, and not cysteine itself, is employed as an antioxidant will be discussed later. In contrast to eukaryotes, GSH is less commonly employed by prokaryotes as an antioxidant defense mechanism (*9, 10*). GSH is often found in Gram-negative bacteria, but rarely in Gram-positive bacteria. However, it is noteworthy that a bewildering array of other thiols are employed by (especially aerobic) bacteria as defenses against oxidative stress (*11-13*). GSH is particularly efficacious in sequestering



GSH

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hypohalites that are involved in human host defense, such as hypochlorite (*14*), hypobromite (*15*), and hypothiocyanite (*16*). For bacteria that possess GSH, it is apparent that GSH is also employed as a defensive measure against oxidants, including those that specifically target sulfhydryl groups (*17*) as well as less discriminate oxidants like hypochlorite (*18*). However, even for bacteria that possess substantial reserves of GSH in their cytoplasms, GSH is apparently not always essential in the defense of bacteria against some oxidative biocides, for example, hydrogen peroxide (*19*). For hydrogen peroxide, one reason why GSH may be dispensable is the fact that in the absence of metal catalysts, hydrogen peroxide and GSH (as well as other derivatives of cysteine) react sluggishly (*20, 21*). The various reactions of hydrogen peroxide compete *in vivo* with the very efficient enzyme-catalyzed disproportionation by catalases: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$, although not all bacteria are catalase-positive (*22*). It is noteworthy that in the parts of the body where the human defense factor hypothiocyanite (a pseudo-hypohalite that is known to react selectively with sulfhydryl groups (*16*)) is generally abundant, such as those regions of the body that are controlled by the mucosa and the exocrine fluids, human commensal bacteria are often capable of synthesizing GSH (e.g., *Escherichia coli* in the gut (*9*) and *Lactococcus lactis* in milk (*23*)). GSH is also employed by some human pathogens (including some Gram-positive species of *Streptococcus and Enterococcus*), presumably to evade human defense factors that are often oxidative in nature. It is remarkable that some human pathogens do not have the capability of GSH biosynthesis, but they instead acquire exogenous GSH from their host for the purpose of defense against oxidative stress. Such species include *Streptococcus mutans* (*24*) and *Haemophilus influenzae* (*25*). *S. mutans* is associated with dental caries in the oral cavity (which is controlled by saliva that contains high concentrations of hypothiocyanite (*26*)). *H. influenzae* is associated with invasive infections of the lungs (which is an environment that is controlled by mucosa that is also believed to contain high concentrations of hypothiocyanite (*27*)). Despite the beneficial role of cysteine derivatives in the defensive stratagem, such compounds have a dark side. Using compounds with relatively reactive moieties like sulfhydryl groups as a defensive mechanism creates the opportunity for alternative host-compromising reaction pathways. However, before discussing these issues that concern cysteine, it is worthwhile to delineate the marked differences that exist between the chemistry of the two biocides hydrogen peroxide and hypochlorite.

Comparison of the Chemistries of Hydrogen Peroxide and Hypochlorite

Hydrogen peroxide and hypochlorite are both oxidative biocides, but they exhibit very different reaction chemistries. Thus, hydrogen peroxide is a

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thermodynamically powerful oxidant, but hypochlorite (or more accurately the conjugate hypochlorous acid) is kinetically more reactive. The reasons for these trends can be explained by examining the differences between the chemical and physical properties of hydrogen peroxide and hypochlorous acid, beginning with their standard reduction potentials:

$$H_2O_2(aq) + 2H^+ + 2e^- = 2H_2O \quad \varepsilon^o\,(1M\,H^+) = \; 1.78\ V \qquad (1)$$
$$HO_2^- + H_2O + 2e^- = 3OH^- \quad \varepsilon^o\,(1M\,OH^-) = \; 0.88\ V \qquad (2)$$

$$HOCl(aq) + H^+ + 2e^- = Cl^- + H_2O \quad \varepsilon^o\,(1M\,H^+) = \; 1.48\ V \qquad (3)$$
$$OCl^-(aq) + H_2O + 2e^- = Cl^- + 2OH^- \quad \varepsilon^o\,(1M\,OH^-) = \; 0.84\ V \qquad (4)$$

It is clear that the conjugate acids of these two biocides are more powerful oxidants than their corresponding conjugate bases (e.g., Eqn 1 vs. 2 and Eqn 3 vs. 4). The first acid dissociation constant (pK_a) of hydrogen peroxide is 11.8; therefore, the predominant form is H_2O_2 at neutral pH. In contrast, the pK_a of hypochlorous acid is 7.4; therefore, comparable amounts of HOCl and OCl^- exist at neutral pH. Nonetheless, interconversion between the conjugate acid and base is rapid (nearly diffusion-controlled (*28*)). Accordingly, the reactive form is HOCl. Using the Nernst equation to compute the reduction potentials at neutral pH, we find H_2O_2 (1.37 V) is thermodynamically a more powerful oxidant than the $HOCl/OCl^-$ equilibrium mixture (1.25 V). This point is relevant in the context of human host defense because myeloperoxidase (MPO, an enzyme that is employed by neutrophils) generates hypochlorite *in vivo* by oxidizing chloride ion with hydrogen peroxide. MPO is the only mammalian enzyme that is capable of effecting this reaction (*29*). Another clear difference that exists between hydrogen peroxide and hypochlorous acid is the polarity of the heavy atom bond in each. Since HO-OH is symmetrical, the bond is not polarized, but this is not the case for hypochlorous acid, for which the bond is best described as $HO^{\delta-}\text{-}Cl^{\delta+}$ (as a consequence of the relative electronegativities of oxygen and chlorine). Finally, it is noteworthy that there is a large difference between the O-O bond enthalpy of hydrogen peroxide (~34 kcal mol^{-1}) and the O-Cl bond enthalpy of hypochlorous acid (~52 kcal mol^{-1}) (*30*). As a consequence of the differences between the reduction potentials, the bond enthalpies, and the bond polarities, hydrogen peroxide and hypochlorous acid tend to react via very different reaction mechanisms. The most kinetically facile reaction pathways of hydrogen peroxide generally involve one-electron mechanisms that yield hydroxyl radicals via homolytic cleavage of the O-O bond, whereas hypochlorous acid invariably reacts via electrophilic/nucleophilic pathways that involve the effective transfer of Cl^+ to the reaction partner. These distinctive reaction pathways of hydrogen peroxide and hypochlorous acid are illustrated in the kinetics and mechanisms of their reactions with cysteine. While hydrogen peroxide can apparently react directly with cysteine in a two-electron process,

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this reaction pathway has a high activation energy, and consequently the reaction rate is slow (*20*). However, hydrogen peroxide produces hydroxyl radicals in the presence of metals in a process that is often referred to as the Fenton reaction (*31*). Thiols are effective scavengers of hydroxyl radicals (*32*). Thus, a general mechanism for the reaction of hydrogen peroxide with a thiol, as catalyzed by metals, is illustrated below:

$$H_2O_2 + M^{n+} \rightarrow OH^{\bullet} + OH^- + M^{(n+1)+} \qquad (5)$$

$$RSH + OH^{\bullet} \rightarrow RS^{\bullet} + H_2O \qquad (6)$$

$$RS^{\bullet} \rightarrow \tfrac{1}{2}\,RSSR \qquad (7)$$

$$\underline{RSH + M^{(n+1)+} \rightarrow \tfrac{1}{2}\,RSSR + H^+ + M^{n+}} \qquad (8)$$

$$H_2O_2 + 2\,RSH \rightarrow 2\,H_2O + RSSR \qquad (9)$$

There is a need for a supply of reduced metal to drive such one-electron chemistry. For Eqns 5-9, that reductant is the thiol (Eqn 8). However, the reductant *in vivo* will be a point of discussion later. Also, it is important to point out that the intimate reaction mechanism for various metals and thiols may be different, since the metal is often incorporated into reactive intermediates (*33-37*). Thus, the "hydroxyl radicals" that are generated in Fenton reactions frequently exhibit different reaction selectivities (*38*). In contrast to the one-electron chemistry of hydrogen peroxide, hypochlorous acid tends to act as a two-electron oxidant vis-à-vis a chlorinated intermediate:

$$RSH = RS^- + H^+ \qquad (10)$$

$$OCl^- + H^+ = HOCl \qquad (11)$$

$$RS^- + HOCl \rightarrow RSCl + OH^- \qquad (12)$$

$$RSCl + H_2O \rightarrow RSOH + HCl \qquad (13)$$

$$\underline{RSOH + RS^- \rightarrow RSSR + OH^-} \qquad (14)$$

$$2RSH + OCl^- \rightarrow RSSR + H_2O + Cl^- \qquad (15)$$

The acid-base equilibria (Eqns 10 and 11) are significant because the maximum rates are achieved for the oxidation of the thiol at a pH in which an equimolar concentration of hypochlorous acid (the better electrophile) and thiolate (the better nucleophile) are present in solution (i.e., the midpoint between the two acid dissociation constants, which is about pH 8 for hypochlorous acid and a typical thiol). Indeed, one reason that thiolates are so readily oxidized by hypochlorous acid is the fact that they are one of the most powerful nucleophiles in a biological setting. Hydrolysis of the intermediate sulfenyl chloride is a facile process in an aqueous environment, as is condensation of the transient sulfenic acid with thiolate to give the disulfide (*39*). However, these highly reactive sulfur species (RSS) are capable of engaging in other reaction chemistries (*vide infra*). Thus far, we have focused on the beneficial role of cysteine as an antioxidant. With reference to the potential injurious qualities of cysteine derivatives, we will next review the well-recognized role of cysteine derivatives in the generation of reactive oxygen species (ROS) and its ability to affect cell physiology. We will then turn our attention to less-celebrated chemistry, that may implicate cysteine in the formation of secondary biocides.

Influence of Cysteine on the Efficacy of Hydrogen Peroxide as a Biocide

The addition of hydrogen peroxide to aerobic cultures of some bacteria is known to cause DNA damage and dose-dependent growth inhibition (*40*). Cysteine has also been shown to inhibit the growth of various bacteria. Under aerobic conditions, no additional oxidant is required for cysteine to inhibit growth, as toxic levels of hydrogen peroxide (and other reactive oxygen species) are apparently formed when the cysteine is oxidized (*41, 42*). When cysteine and hydrogen peroxide are used in combination, enhanced toxicity is observed (*43-45*). This is a curious observation, in that hydrogen peroxide is a powerful oxidant and cysteine is readily oxidized, nevertheless, as explained earlier, the direct reaction of these two species is a kinetically slow process (Figure 1, Rxn 1). However, it has been known for nearly a century that free metal ions (in particular iron) are known to catalyze the oxidation of cysteine by hydrogen peroxide (Figure 2, part of the catalytic cycle is illustrated in Rxn 2) (*46*), although the mechanism itself can be very complicated. In the absence of cysteine, as discussed earlier, it is well-known that iron, as well as other transition metals, are capable of homolytically cleaving the O-O bond to give a hydroxyl radical (Figure 1, Rxn 3), and that the hydroxyl radical is known to react rapidly with cysteine to eventually give cystine (Figure 1, Rxn 4). However, the hydroxyl radical exhibits promiscuous chemistry, and it is known to react with a great many species with near diffusion-controlled kinetics (*47*), including cysteine (*48*) and glutathione (*49*). DNA is particularly susceptible to damage by hydroxyl radicals because bacterial chromosomes occupy a significant volume of the cytoplasm (*50*), because diffusion is restricted by the densely-packed contents of the cytoplasmic vesicle (*50*), and because iron is known to bind to DNA (thereby increasing the probability that hydroxyl radicals will be generated in the vicinity) (*51*). As explained earlier, Fenton chemistry requires a steady supply of reduced metal. In the case of iron, this is the divalent oxidation state. At neutral pH in the absence of an applied potential, thermodynamics predicts an equilibrium mixture of $Fe^{II}(OH)_2$ and $Fe^{III}(OH)_3$. However, in an oxygenated environment, Fe(III) is the preferred oxidation state. It is noteworthy that in the absence of chelating ligands, Fe(III) is highly insoluble at neutral pH (*52*). Its insolubility in an aerobic aqueous environment is one of the reasons that iron is a precious commodity that is guarded by living organisms (for ferric hydroxide, $K_{sp} \approx 10^{-38}$) (*53*). Accordingly, there is generally relatively little "free" (i.e., Fenton-active) iron *in vivo* (ca. 20 μM in the

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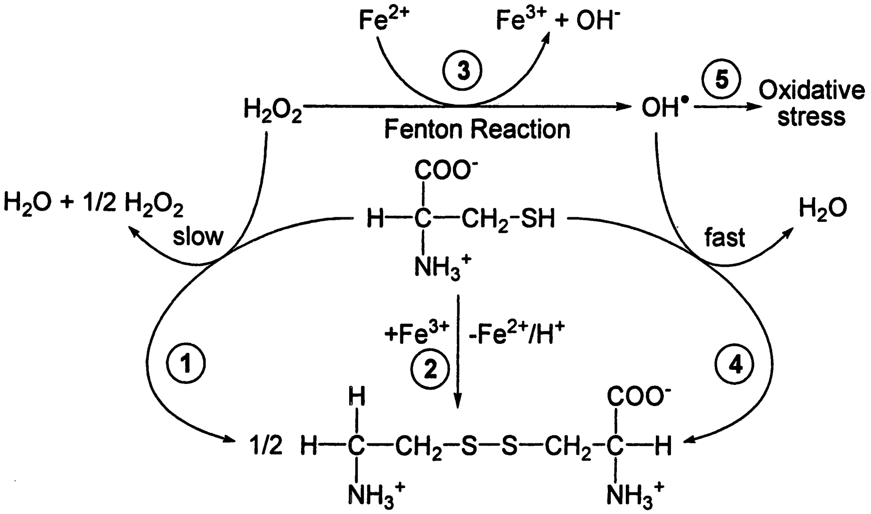

Figure 1. Proposed dual roles of cysteine as an antioxidant and as a reductant of iron(III) that support deleterious Fenton chemistry.

cytoplasm of *E. coli* (*54-56*)). Once depleted in a Fenton reaction to produce Fe(III), it is necessary to reduce the iron back to the divalent state to drive the reaction. It has been suggested that the role of added cysteine (with or without added hydrogen peroxide) is to reduce the Fe(III) to Fe(II), thereby facilitating Fenton chemistry (*44, 57, 58*). In support of the hypothesis that insult by exogenous cysteine (and cystine) promotes intracellular Fenton-mediated oxidative damage are the observations that:

1. Cells that are cultured with poor sulfur sources, and subsequently exposed to cystine, exhibited transiently greater sensitivity to hydrogen peroxide (*44*).
2. DNA filamentation is associated with killed cells, and a high rate of mutation is observed amongst those that survived the insult (*44*).
3. Cell-permeable iron chelators ameliorated the damage (*44*).
4. Hydrogen peroxide sensitivity is associated with the transport of cysteine into the cell (in a process that is coupled with export of GSH out of the cell to facilitate reduction of the cystine (*58*)) as indicated by the fact the L-cystine is effective at sensitizing the cells, while D-cystine is not (*44*).
5. Mechanisms that suppress the enzymatic reduction of free cystine are capable of restraining Fenton chemistry that is driven by cysteine (*59*).

It would appear that there is substantial evidence that cysteine is capable of promoting oxidative stress. However, the oxidation potential of GSH is very similar to cysteine (*60*). Why then is Fenton chemistry not facilitated in the cytosols of cells that contain millimolar concentrations of GSH? One explanation could be that the kinetics are unfavorable. However, model systems that are comprised of Fe/ETDA/H_2O_2 and either cysteine or glutathione are able to hydroxylate salicylate (a standard analytical model for measuring the generation of hydroxyl radicals) at a rate that appears to be within an order of magnitude (*61*). And yet, as discussed earlier, living cells that contain millimolar concentrations of GSH are oxidatively stressed upon addition of cysteine (*44, 45, 58*). Significantly, *in vitro* experiments have shown that cysteine is capable of driving Fenton-mediated DNA damage, but, under identical conditions, GSH does not *(44)*. Given the fact that the metal and the thiol play an intimate role in the kinetics of the Fenton reaction, and it is highly likely that the metal is associated with the biological molecules that are damaged by the hydroxyl radical that is produced, a general hypothesis could be advanced that a special arrangement of ancillary ligands favors the involvement of cysteine for Fenton-active iron *in vivo*. However, this scenario appears unlikely to us. Furthermore, the previous suggestion (a specific hypothesis) that cysteine is more effective than GSH in driving Fenton chemistry, due to preferential complexation of cysteine with iron, is not likely to be correct (*44*). In fact, GSH is capable of competing effectively with enterobactin (*62*), a siderophore that is employed by bacteria for the purpose of iron acquisition (*63*). Furthermore, there have been quantitative mechanistic studies of the reduction of Fe(III) by both cysteine (*36, 37*) and GSH (*33-35, 64*) which suggest that the rates of complexation and the subsequent reductive eliminations are comparable. Since the aforementioned *in vitro* experiments that involve the kinetics of reduction of Fe(III) (i.e., references (*33-37, 64*) vs. (*44*)) and the quantification of Fenton-induced damage (i.e., references (*61*) vs. (*44*)) appear to be incongruous, additional investigation of this phenomenon appears to be warranted. Furthermore, it is likely that the insult by unnaturally large concentrations of cysteine induces physiological stress, and it is unclear what role if any such stress plays in contributing to the cytotoxicity of cysteine. It is conceivable that alternative, perhaps unrecognized, stress-inducing mechanisms are activated by cysteine. We will briefly discuss some issues that pertain to the topic of cysteine-induced physiological stress next. First, we will address possible non-Fenton-induced oxidative stresses; then we will turn our attention to the inhibitory effects of cysteine on certain biosynthetic pathways.

Influence of Exogenous Cysteine and Cystine on the Redox Status of *E. coli*

Detailed investigations of the role of cysteine in oxidative stress have thus far focused on *E. coli*, a Gram-negative bacterium. Like many Gram-negative

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bacteria, the cytoplasmic potential in *E. coli* is maintenanced by balancing the reduced and oxidized forms of redox molecules including GSH/GSSG. The redox potential of the periplasmic space of *E. coli* is also tightly regulated. An ATP-binding cassette (ABC) transporter has been recently identified in the cytoplasmic membrane of *E. coli* that exports GSH (but not GSSG) to the periplasm (CydDC) (*65*). CydDC has also been shown to transport cysteine (but not cystine) (*66*). One purpose for the export of these reducing agents to the periplasm of *E. coli* could be the maintenance of disulfide bond reductases, oxidases, and isomerases (Dsb enzymes) that play key roles in protein folding in the periplasm (*67*). Also, CydDC has been recently implicated in the cytotoxicity of cystine (in the presence of hydrogen peroxide) in that exogenous GSH is required to reduce cystine to cysteine before transport into the cell (*58*). Since the outer membrane is freely permeable to small molecules like cysteine and GSH (*68, 69*), the addition of exogenous thiol or disulfide should also be capable of rapidly perturbing the redox status of the periplasm. Indeed, it has been previously shown that exogenous cysteine and GSH (reducing agents) are capable of rescuing mutants that lack the ability to synthesize CydDC (*70*). It is also proven possible to reverse the effects of strains that are deficient in Dsb enzymes that form disulfide bonds by adding exogenous cystine (*71*). Accordingly, it is clear that both exogenous cysteine and cystine influence the redox status of the periplasm. But, can cysteine and cystine induce oxidative stress? Actually, there is sound evidence for "disulfide stress" in *Streptomyces coelicolor* (*72*). Given the fact that the trafficking of GSH and cysteine are tightly controlled by the inner membrane of *E. coli*, it would appear unlikely that exogenous thiol/disulfide would have a substantial impact on the redox status of the cytoplasm (at least immediately). However, the periplasm is another matter altogether. Given the facts that small molecular thiols/disulfides freely diffuse through the outer membrane (*68, 69*), that thiols are apparently employed to maintain redox homeostasis in the periplasm (*65, 66*), that periplasmic enzymes exist to rapidly establish thermodynamic equilibrium between thiols and disulfides (*67*), and given the likelihood that two component signal transduction pathways exist that are capable of sensing redox imbalance in the cell envelope (*73*) and activating stress factors in the cytoplasm (including the alternative sigma factor RposS (*74*)), it appears very probable to us that the addition of exogenous cystine and cystine solicite a stress response. While such a scenario would explain the expression of antioxidant genes upon insult by cystine (*58*), and it might explain the growth inhibition effect of cysteine, it would not necessarily explain the DNA damage that is known to occur in the cytoplasm upon insult by cysteine (*44*). Furthermore, it is generally believed that it is the DNA damage that occurs when *E. coli* is exposed to hydrogen peroxide that leads to cell death (*75, 76*). Fenton chemistry could indeed explain nuclear damage, but other explanations may be possible. For example, it is recognized that a sudden arrest in growth can induce oxidative stress and the production of

ROS (*77*). However, we are not aware of evidence that such stress induces nuclear damage. Nonetheless, we believe additional investigation of this topic is warranted.

Inhibition of Biosynthesis by Cysteine

In addition to the abovementioned evidence that cysteine is capable of facilitating the infliction of oxidative damage, it is well-recognized that cysteine is capable of inhibiting biosynthesis, and especially branched-chain amino acid synthesis (*78-82*). For example, cysteine is known to inhibit threonine deaminase, a key enzyme of the isoleucine, leucine, and valine pathway (*80, 83*). Accordingly, the inhibition of *E. coli* that is caused by cysteine can be overcome by the addition of leucine and isoleucine to the growth medium (*80*). Similar results have been obtained for oral *Streptococci* (*82*). Importantly, the *E. coli* cultures were grown aerobically, and no effort was made to differentiate between the effects of oxidative damage and the effects of suppression of biosynthetic pathways. However, the oral *Streptococci* were grown anaerobically. Accordingly, it is possible that the observed effects were due to suppression of biosynthetic pathways alone. However, we note that some oral *Streptococci* (including the strain of *Streptococcus mutans* that was employed in the aforementioned study) are catalase negative. Accordingly, hydrogen peroxide is generated if such facultative (aerotolerant) anaerobes are grown in the presence of oxygen (*84*). Surprisingly, Fenton-induced oxidative damage has also been observed when these organisms are grown under strict anaerobic conditions (*85*). Accordingly, it is not clear what the significance is between the repression of biosynthetic pathways and the observed cysteine cytotoxicity. Furthermore, since physiological stress is known to both enhance and diminish the sensitivities of various microorganisms to biocides, it is unclear what effect, if any, the physiological stress that is induced by cysteine might have on the efficacy of biocides, including the aforementioned hydrogen peroxide.

Why is Glutathione and not Cysteine Employed as a Redox Buffer?

Oxidative stress and the inhibition of biosynthesis by cysteine aside, a simple explanation exists as to why cysteine is not employed as an intercellular reducing agent: solubility. At pH 7 and an ionic strength of unity, cystine has a solubility of only about 1 millimolar (*86*). L-Cystine is the least soluble of the amino acids. Thus, under conditions of oxidative stress, millimolar concentrations of cysteine, once oxidized, would begin to precipitate. In fact,

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cystine stones in humans, which are caused by a genetic disorder, account for a significant percentage of all urinary stones (*87*).

Influence of Cysteine on the Efficacy of Hypochlorite as a Biocide

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As was the case for hydrogen peroxide, GSH is known to protect certain bacteria from oxidative stress that is induced by hypochlorite (*57, 88, 89*). Furthermore, hypochlorite is known to promote Fenton-like chemistry with the generation of hydroxyl radicals (*90*). Thus, many of the same issues that were discussed previously for the involvement of cysteine and hydrogen peroxide are pertinent to the biocidal properties of hypochlorite. However, whereas Fenton chemistry is of prominent importance for hydrogen peroxide, other reaction pathways probably dominate in the case of hypochlorite (*vide supra*). Perhaps the most significant characteristic that distinguishes the biocidal properties of hydrogen peroxide and hypochlorite is the fact that the former oxidant is relatively kinetically inert, whereas hypochlorous acid reacts with a variety of biologically-significant functional groups with kinetics that approach the diffusion-controlled limit. In particular, as was discussed by one of us in another chapter in this volume, hypochlorous acid is particularly reactive toward derivatives of cysteine (*14, 91, 92*) and methionine (Met) (*93*). Because of the high reactivity of hypochlorous acid, the lifetime of this biocide in the mélange of a typical application is very short. One pragmatic consequence of the rapid reaction of hypochlorous acid with organic compounds in the testing of biocides that react rapidly and indiscriminately is the fact that the nature of the medium and the protocol of mixing can have a pronounced effect on the reliability of the assay (*94*). While the importance of the nature of the medium (often referred to as the "organic load") is well-recognized (*94-97*), and in fact is one of the variables often taken into consideration when designing an assay that is intended to probe the efficacy of a biocide under conditions of normal use, the issue of turbulent mixing has apparently been overlooked. We will next illustrate the challenge of reproducible mixing when performing biocidal assays with hypochlorite.

The AOAC 955.16 method for measuring available chlorine in disinfectants is required by the EPA Office of Pesticide Programs for DIS/TSS documentation of certain applications. While it is a specific protocol, it is illustrative of a general problem with most bacterial suspension tests that involve highly reactive disinfectants like hypochlorite. Dilutions of 200, 100, and 50 ppm available chlorine in 10 ml pH 7 buffer are prepared and challenged by adding a 50 μl inoculate of ca. 10^9 cells/ml of a bacterium (typically *Salmonella typhimurium*, for an initial concentration of ca. 5×10^6 cells/ml). At set intervals of 90 seconds, the solution is subcultured at the 60 second mark, and an additional 50 μl of inoculate is added at the 90 second mark, until a total of 10x50 μl of culture have been added. The culture medium that is employed can either be an undefined medium (nutrient broth, NB) or a defined synthetic broth that consists of various amino acids, and vitamins. We have separately measured the rate of reaction of 200 ppm NaOCl with the defined medium and *E. coli* cells. The reaction with the cells exhibits the rate law $-d[OCl^-]_T/dt=k[OCl^-]_T[cell(number/ml)]$, where $k=3.2(3)\times10^4$ $M^{-1}s^{-1}$ under pseudo-first-order excess of hypochlorite ($[OCl^-]_T=[HOCl]+[OCl^-]$). The half-life of the reaction of hypochlorite with the cells, after adding the first 50 μl of culture to a 200 ppm (2.68 mM) solution of hypochlorite is 12 ms. We have observed in a separate experiment that the defined synthetic medium (without cells) reacts via three distinct kinetic steps during the first 90-second time interval. About 7% of the hypochlorite reacts with a half-life of 3 ms. Another 20% of the hypochlorite reacts with a half-life of about 10 seconds. The third event is not complete by the time the second 50 μl inoculum is added, but a total of ca. 31% of the hypochlorite has been consumed by the medium alone before the second inoculate is introduced to the test mixture. Thus, by the fourth inoculum, all of the hypochlorite has been consumed by the medium alone, presumably to form secondary biocides. The problem is more acute for the 50 ppm solution of hypochlorite, for which all of the hypochlorite is consumed before the second inoculum is added! It is important to point out that the cells in this protocol are kinetically competent to react directly with the hypochlorite (at least for the first inoculate), but only in the presence of the secondary species that are formed when the hypochlorite reacts with components in the medium during the first few milliseconds of the reaction. There are two other, more subtle issues that come to light regarding this example. First, although a new inoculate is introduced every 90 seconds, there are still less reactive secondary derivatives of the previous inoculate that remain unreacted at the point that the next inoculate is introduced. These components are expected to accumulate to the point that

Table 1. Typical HOCl biocide assay by tube dilution illustrating precision and reproducibility when rapid mixing is employed (10^7 *E. coli*/ml).

$[HOCl]_0$ (μM)	rapid	pipette
1000	-	-
500	-	-
250	-	-
125	-	-
63	-	-
60	-	-
55	-	-
50	-	-
45	-	-
40	-	+
35	-/-	+/-
30	+	-
25	+	-
20	+	-
15	+/+	-/+
10	+	+
8	+	+
4	+	+
2	+	+
1	+	+

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they become the dominant reactants. The other effect is even more insidious. For diffusion-controlled reactions, if turbulent mixing conditions are not employed, heterogeneous reaction may occur. We have recently demonstrated this point in a study of the reaction of hypochlorite with cystine (*98*). Failure to employ turbulent mixing results in local high concentrations of hypochlorite that react before manual mixing can be achieved. Without turbulent mixing, cells can experience locally high concentrations of hypochlorite, perhaps influencing the assay. Indeed, we have carried out several experiments that evidence the influence of mixing rates. For example, the data in Table 1 are for the mixing of $1x10^7$ E. coli cells (washed 2x and suspended in phosphate buffered solution, PBS) with a serial dilution of hypochlorite. Experiments that involve pipetting equal volumes of culture and hypochlorite solutions, followed by brief vortexing, invariably require more HOCl/cell to completely inhibit growth than that required for even modest turbulent mixing (in this case ca. 1 ml/s). Furthermore, near the minimum inhibitory concentration (MIC), the results are not reproducible unless turbulent mixing conditions are employed (Table 1, 2-fold dilutions in bold, and duplicate measurements indicated).

Given the fact that under conditions of heavy organic load most of the hypochlorite will react with the medium before it interacts with the bacterial cells, the issue naturally becomes: what is actually serving as the biocide? In the context of the focus of this chapter, Figure 2 illustrates the reactions that are observed when hypochlorous acid is reacted with cysteine. The first reaction produces the sulfenic acid (presumably vis-à-vis a sulfenyl chloride, as was illustrated previously in Eqns 12 and 13). Sulfenic acids are very reactive species, particularly in aqueous medium. In the presence of large excesses of cysteine, cysteine sulfenic acid produces cystine (Eqn 14). However, we have observed that even in the presence of a 20-fold excess of cysteine, large amounts of the thiosulfinate ester are produced. In fact, this is the first product to be observed (on the millisecond timescale) when cysteine is oxidized by hypochlorite in the pH range of 10 to 14. We have not yet investigated the initial products of the oxidation of cysteine by hypochlorous acid at physiological pH, but we believe that previous reports that the sulfenyl chloride is relatively long-lived are incorrect (*99, 100*). The thiosulfinate ester that is produced is a RSS that undergoes pH-dependent hydrolysis. Nonetheless, its transient lifetime is significant at neutral pH. The thiosulfinate ester moiety is significant in that it is represented in allicin, a powerful antibiotic and anti-fungal compound obtained from garlic. Allicin is enzymatically produced from a stable precursor upon injury to the plant (*101*). Like the thiosulfinate ester of cysteine, allicin is hydrolytically unstable. Similar compounds have also been investigated as antimicrobials (*2, 3*); therefore, it is reasonable to suppose that the thiosulfinate ester of cysteine might also possess antimicrobial properties. Our first efforts to demonstrate such antimicrobial behavior have not been met with success, in that

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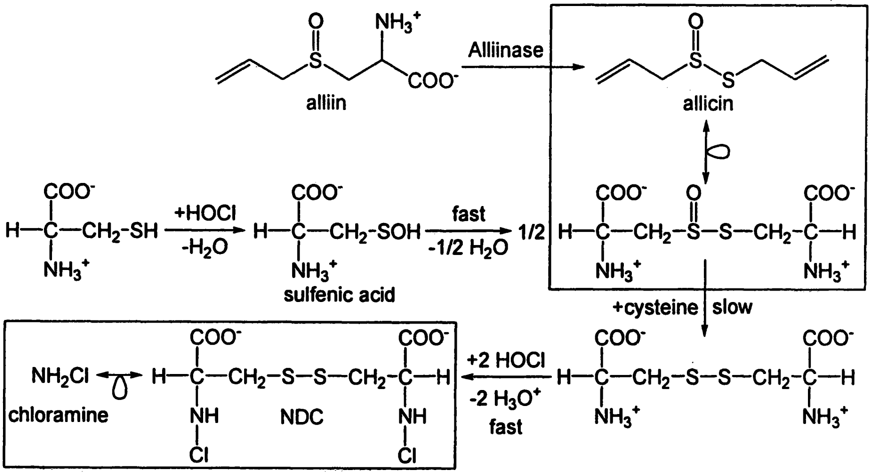

Figure 2. Oxidation of cysteine by hypochlorous acid to give intermediates with potential biocidal properties. The observed transient thiosulfinate ester is analogous to the biocide allicin. The observed chloroamine derivative of cystine is chemically related to the biocide chloramine.

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suspensions of *E. coli* in PBS were unaffected by millimolar quantities of the thiosulfinate ester of cysteine. The fact that exogenous thiosulfinate ester is not cytotoxic is probably not surprising, because it is likely that cysteine (and not cystine) is transported into the cell (*66*), so active transport of the analogous thiosulfinate ester seems unlikely. Furthermore, passive diffusion is not likely to take place in the timeframe of the stability of the thiosulfinate ester at neutral pH. Although the thiosulfinate ester does not apparently play a role *in vitro*, it may nonetheless still be relevant to the chemistry that takes place *in vivo* (*2, 102, 103*).

We are currently investigating the biological chemistry of the thiosulfinate ester of cysteine. In the presence of excess cysteine, the thiosulfinate ester undergoes further reaction with excess thiol to give disulfide in a reaction that is apparently competitive with hydrolysis (as evidenced by the rate law we have observed for the formation of cystine from the thiosulfinate ester in the presence of excess cysteine). Regardless of whether cystine is produced by capture of the transient sulfenic acid by cysteine or by the nucleophilic attack of cysteine on the more stable thiosulfinate ester, the end product is cystine (Figure 2). In the presence of excess hypochlorite, we have observed the formation of transient chloroamine derivatives of cystine (*98*). This observation contrasts with previous reports that hypochlorite attacks the disulfide bond of cystine (*14*). We have also observed that oxidized glutathione produces the bis-N-dichloro-γ-L-glutamyl derivative (ClGSSGCl). We have also carried out preliminary toxicity studies of the chloroamine derivative of ClGSSGCl with *E. coli*. As with the sulfinate ester, these preliminary studies have proven inconclusive. However, we note that the parent chloramine compound (Figure 2) is frequently employed as a biocide, as are N-alkyl and aryl derivatives. Nonetheless, we have observed that ClGSSGCl is unusually stable. If there is a biochemical pathway to reduce ClGSSGCl to GSH, such a process would allow GSH to buffer three times the amount of hypochlorous acid than it is presently given credit for. Thus, instead of buffering a one-half molar equivalent of hypochlorous acid (Eqn 16), each mole of GSH would buffer one and one-half molar equivalents of hypochlorous acid (Eqn 18):

$$\text{GSH} + \tfrac{1}{2}\,\text{HOCl} \rightarrow \tfrac{1}{2}\,\text{GSSG} + \tfrac{1}{2}\,\text{H}_2\text{O} \qquad (16)$$

$$\underline{\tfrac{1}{2}\,\text{GSSG} + \text{HOCl} \rightarrow \tfrac{1}{2}\,\text{ClGSSGCl} + \text{H}_2\text{O}} \qquad (17)$$

$$\text{GSH} + 1\tfrac{1}{2}\,\text{HOCl} \rightarrow \tfrac{1}{2}\,\text{ClGSSGCl} + 1\tfrac{1}{2}\,\text{H}_2\text{O} \qquad (18)$$

Chesney *et al.* have observed an LD_{50} for a GSH-deficient strain of *E. coli* that is about two times lower than that of the WT (*18*). Since the strains are otherwise isogenic, this difference in survival is attributed to GSH stasis. Remarkably, exogenous GSSG fully restored resistance of the GSH-deficient strain toward hypochlorous acid *(18)*, which we attribute to the formation of ClGSSGCl, a chloramine that is apparently not cytotoxic. Since 95% of the cells survived when 70% of cytoplasmic GSH is oxidized (and 50% survived when 150% was oxidized!), this raises the issue of whether ClGSSGCl plays a role *in vivo*. We are currently examining possible enzymatic pathways that might be employed to reduce ClGSSGCl.

Conclusions

We have presented a progress report of our studies of hydrogen peroxide and hypochlorite with a particular emphasis upon the role cysteine plays in attenuating their biocidal properties. In keeping with the tradition of the ACS Symposium Series, we have made a point of stressing the current state of the topic. This is not a subject that lacks complexity. There are many questions that remain unaddressed, and in many cases the existing data are not self-consistent. In particular, there seem to be inconsistencies between experiments that have been carried out *in vitro* and *in vivo*. The relationship between hydrogen peroxide and cysteine has received considerable attention. Particularly worrisome is the disconnect that appears to exist between the rates of Fenton reactions with small molecules under pristine conditions, related reactions that occur with macromolecules, and the kinetics that are inferred within cells. These studies illustrate the challenges that exist in developing mechanistic models for

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events that occur in the complex milieu of the inner cell. However, it is perhaps these inconsistencies that deserve the most attention, since they speak to chemistries that are not well understood. In some cases, the fundamental chemistry of the small molecules has not been sufficiently developed such that we are prepared to extend our outlook to the biological chemistry. We are currently at the stage in the case of the chemistry between the hypohalites and cysteine derivatives of developing an understanding of the kinetics and mechanisms of the reactive intermediates that are generated. While such chemistry is mature in non-aqueous solvent, much less is known about such intermediates in an aqueous environment.

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Chapter 11

Identification Strategy of Mechanism-Based Lipophilic Antimicrobials

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The dependency between antimicrobial activity and lipophilicity (logP) of monooxygenated alklyphenols (MOAPs) was studied for a greater number of bacterial and fungal species from literature data., which was found to be significant for all species examined. The antimicrobial activity is explained with unspecific cytoplasmic membrane damaging effects that is in optimum at a lipophilicity ($\log P_0$) of 4 (±0.5) for gram-negative bacteria, at $\log P_0$ of 5 for fungi and at $\log P_0$ of 6 (±0.5) for gram-positive bacteria. Antimicrobials with reported mechanism of action, e.g. MOAPs with complex carbon skeleton or azole antifungal drugs, separated clearly from the data points of unspecifically acting compounds in regression analysis. It was therefore concluded that the regression lines calculated for unspecifically acting MOAPs are useful as standard curves in the analysis of microbiological inhibitory data. This method allows a systematical and computer-aided selection of potential mechanism-based inhibitors. In addition, three types of microbial inhibitors are classified: membrane damaging compounds ($\leq \log P_0$), compounds desorbing from the cytoplasmic membrane ($\log P_{\text{max.}} \leq 7.5$ to 8), and compounds selectively acting in the cytoplasmic membrane or cell wall ($\log P > 8$).

1. Introduction

In the finding of new antibiotic leads, limitations of fundamental chemical and physical laws prevent the direct quantification of biological activity. To face the problem several antibiotic-likeness models have been successfully developed that e.g. allow the identification of antibiotics by use of compound descriptors. Although new anti-infective leads could be introduced by rules that have been deduced from QSAR models of well-studied traditional antibiotics, really new structures were not identified (*1*).

On the contrary thousands of compounds of natural and synthetic origin have been examined on their antimicrobial properties according to different testing protocols. However, a strategy - other than searching for compounds having low minimal inhibitory concentrations (MIC) - for the evaluation of the observed activities does not exist. In this work an attempt was done to find out a strategy how to distinguish between unspecific and specific acting antimicrobials.

It is known that phenols solute inside the cytoplasmic membrane of cells in proportion to their lipophilicity (*2*) and causes damages, membrane lysis and finally cell death in dose-dependent manner (*3*). This process is governed by both, the physical properties of a given compound plus cytoplasmic membrane and can be summarized as unspecific mode of action. In contrast, specifically acting compounds interfere in biochemical processes and possess a defined target.

In a previous work the inhibitory activity of phenolic phenylpropanes was compared with a series of simple phenols, in which the antimicrobial activity increased significantly with lipophilicity in regression analysis. By this procedure some phenolic phenylpropanes became obvious having clearly higher antimicrobial activities than it could be predicted from their lipophilicities, respectively. It was concluded that beside lipophilicity further factors influence the antimicrobial properties of phenolic phenylpropanes (*4*). A mechanism-based, so far unknown mode of antimicrobial action of selected phenylpropanes seems to be probable.

However, a systematic use of such a strategy for the identification of mechanism-based antimicrobials is untested for a greater number of microorganism species and compounds. Therefore, data of 16 microbial species and over 100 MOAPs are studied in this work and as a consequence regression lines and their validities are calculated. Such compounds follow most simple structural principles to avoid electronic forces caused by additional oxygen or hetero atom substitution at the aromatic ring, which markedly influences antimicrobial activity (*5*).

Partition coefficients have been introduced as a measure for lipophilicity in the interpretation of the pharmacological action of narcotics (*6*,*7*). Both, the antimicrobial and narcotic activity of `indifferent' organic antiseptics (e.g.

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phenol, cresols, and thymol) were found to be related to their solubility in cellular lipoids - in difference to narcotics, antiseptics react chemically with cellular proteins (*8*). By this, it became obvious that physical parameters are useful to explain or even to predict biological properties of organic compounds.

At early times phenol itself was used as surgical antiseptic (*9*). The discovery of new routes in the synthesis of phenols, e.g. Friedel-Crafts-alkylation (*10*) and Claisen-rearrangement (*11*) enabled the production of derivatives with higher antimicrobial activity (*12-16*). Antimicrobial activity of phenols turned out to be related to water solubility (*17*), solubility in olive oil/water (*18*) or hexane/water mixtures (*19*).

The use of logP was introduced by Hansch in the analysis of biological activities of organic compounds (*20*). In this examination a dependency of inhibitory activity from logP of 35 phenols was found for the gram-positive bacterium *Micrococcus pyogenes var. aureus* (*Staphylococcus aureus*), while results obtained with *Salmonella typhosa* (*S. typhi*) were more difficult to interpret due to the different building up of the cell wall of gram-negative bacteria. Lien and Hansch (*21*) concluded that the optimum logP is 4 for gram-negative and 6 for gram-positive bacteria, following correlation analysis with literature inhibitory data of various compound groups towards twelve species of gram-positive and eight species of gram-negative bacteria. In this examination a total of 84 phenols having a logP range of 0.5 to 7.3 were considered; 35 of them were monoethers of benzenediols, the others were halogenated alkylphenols.

2. Method

Inhibitory data of MOAPs obtained in serial and agar dilution tests (minimal inhibitory concentration = MIC, non-inhibitory concentration = NIC, all data given in µg/ml) were taken from computer database on antimicrobials (*22*). In cases of double or multi determinations found in literature preferably the lowest reported MIC was used for the calculations. The MICs were recalculated in molar concentrations and were then used as log 1/MIC ('lgMIC') in regression analysis, respectively (*20*). Octanol/water partition coefficients (CLogP) were calculated as described by Leo (*23*) and is specified as 'logP' in this paper. Calculation and display of regression lines were done with MMP (*24*). The data of n-primary aliphatic alcohols were used to determine $\log P_0$ additionally (*25*).

The microorganism groups are divided in gram-positive (*Bacillus cereus, Bacillus subtilis, Enterococcus faecalis, Listeria monocytogenes, Micrococcus luteus, Propionibacterium acnes, S. aureus, S. epidermidis, Streptococcus mutans)*, gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, S. typhi*) and fungi (*Aspergillus niger, Trichophyton mentagrophytes, Candida albicans*).

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Data points of filled geometric symbols were excluded from regression analysis in Figures **1** to **16**; the compounds included are shown as black circles (○). Figure **17** displays the chemical structures of compounds having complex structures; other compounds are given with their chemical nomenclature only.

3. Results

The data of MOAPs are scheduled together in Tables **I** to **XVI**. Every microbial species is controlled systematically for several criteria to characterize the data situation on which the findings are based. Three types of MOAPs were excluded from regression analysis due to their non-regular behavior. Such compounds are specified with filled data points in the Figures **1** to **16**.

1. inactive: data points shown as filled dots (●) close to x-axis.
2. low activity: data points shown as filled squares (■).
3. high activity: data points shown as filled triangles (▲).

Among MOAPs having high activity, it was distinguished between compounds with unknown and suggested mechanism of action as given Tables **I** to **XVI**. The decision to exclude compounds from regression analysis was a main difficulty. Although MOAPS are built up simple, the data of some members with complex carbon skeleton were surprising. Exclusion was done in the case of existing literature reports on specific mechanism of action of a given compound. A few other compounds were also excluded, because the data obviously didn't fit, which were 4-alkyl-2-benzylphenols (Table **V,VII,X,XII**), ortho-tert.-butylphenols (Table **IX**), 6-alkylnaphthols (Table **XIII**) and phenylphenols (Table **XIV**).

3.1. Gram-Positive Bacteria

The cell envelope of gram-positive bacteria consist of capsule, cell wall and cytoplasmic membrane. The capsule is composed of polypeptides (polyglutamic acid) or various kinds of polysaccharides (amino sugars, glucose, uronic acids, glucuronic acids, N-acetyl-glucosamine). The cell wall has a thickness of 15 to 80 nm and is built up with several layers of hydrophilic peptidoglycan (>50% dry mass, monomeric XlogP = -10.248, (*26*)), teichoic and lipoteichoic acids, lipids and lipoproteins (up to 3%), while proteins and lipopolysaccharides are absent.

Molecules up to a molecular weight of 50 kDa are able to pass through the peptidoglycan (murein) network (*27*). The assembly of peptidoglycan is mediated by a group of enzymes (transglycosylases, transpeptidases and car-

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boxypeptidases), which are localized in the cell wall outside the cytoplasmic membrane. A blocking of those enzymes by penicillin causes block of the assembly of peptidoglycan.

The cytoplasmic membrane allows the permeation of molecules having molecular weights of less than 100 daltons. It is composed of 40% phospholipids (e.g. 2-aminoethoxy-((2R)-2,3-dihexadecanoyloxypropoxy)-phosphinic acid: XlogP = 10.499, (*26*)) and 60% proteins, of which the latter carries out most of the membrane functions, e.g. energy generation, biosynthesis and transport (*28*). The membrane lipids are uniformly composed of saturated or monounsaturated fatty acids (myristic, palmitic, stearic and cis-9-hexadecenoic acid - logP range = 6.1 to 8.2) in most of bacteria, which normally does not allow any conclusion on the identity of a bacterial species by the fatty acid composition (*29*).

If compared to thin layer chromatography the movement of molecules through the outer regions of the envelope of gram-positive bacteria is best for lipophilic compounds having considerable water solubility, because adsorption at the stationary phase (polypeptides, polysaccharides and peptidoglycan with monomeric XlogP ~ -10) is negligible. It takes place after a lipophilic compound has passed through the cell wall and has arrived at the cytoplasmic membrane. Here conditions are comparable to reversed phase chromatography with hydrophobic phospholipids (monomeric XlogP ~10) as stationary phase. Since the mobile phase is still water, the compound desorption renders difficult from the hydrophobic matrix and, in consequence, the lipophilic compounds enrich within the stationary phase or in other words: within the cytoplasmic membrane. Depending on their physical properties the compound still underwent a continuing process of de- and adsorption and passes by this through the membrane to enter the cytoplasma.

The preferred appearance of the more hydrophilic phenol in the cytoplasma of *E. coli* cells points to a stronger adsorption of the more lipophilic ortho-phenylphenol (logP = 3.2) in the cytoplasmic membrane at the MIC (*4*). When entered the cytoplasmic membrane, lipophilic compounds alter it's fluidity at sub-MIC doses (*30*) and they may disturb the integrity of the membrane. In higher amounts a compound solutes in the cell membrane, causes delocalization of proteins, at least disruption and loss of intracellular cations (*31*). Enrichment and damaging effect is parallel with increasing lipophilicity for unspecifically acting compounds, such as phenols and aliphatic alcohols (*19,32*).

An optimum lipophilicity value of logP = 6 was outlined for compounds acting against gram-positive bacteria on the whole (*21*), which might be due to the similar composition of cell walls and cytoplasmic membrane (logP range = 6.1 to 8.2 for free fatty acids).

The inhibitory data of MOAPs towards nine species indicate that almost all of them are toxic to gram-positive bacteria. The lipophilic optima of MOAPs differed not much from the reported optimum of 6 for unspecific acting compounds: *P. acnes* (log P = ≥5.5 to 7), *E. faecalis* (5.5 – 6.5), *S. aureus*

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(~6.5), *B. cereus, B. subtilis, M. luteus* (~6), *S. epidermidis Str. mutans* (≥5.5), and *L. monocytogenes* (log P = ≥3.5 to ≥5.5).

3.1.1. Bacillus cereus

LogP coverage: phenols with similar MIC/logP data pairs are included in this analysis. Data of highly lipophilic phenols (logP >6) isn't available (Table I).

Correlation quality: high dependence ($r^2 = 0.869$) of antimicrobial activity from lipophilicity.

$LogP_0$ definition: ~6. Activity of MOAPs and aliphatic alcohols increased up to a logP of ~6 (Figure 1). This definition is supported by data of aliphatic alcohols.

Comparison with aliphatics: strong activity of tetradecanol (MIC = 40 μg/ml, logP = 6.1); inactivity of pentadecanol (MIC >1000 μg/ml, logP = 6.6 (*83*).

Eq: $\log 1/MIC = 0.671 + 0.728 * \log P, n = 32, r^2 = 0.869, s = 0.346$ (1)

3.1.2. Bacillus subtilis

LogP coverage: the calculated phenols cover logP values from 1.5 to 9 (Table II). Examples of phenols having logP values from 3.5 to 5 are not available.

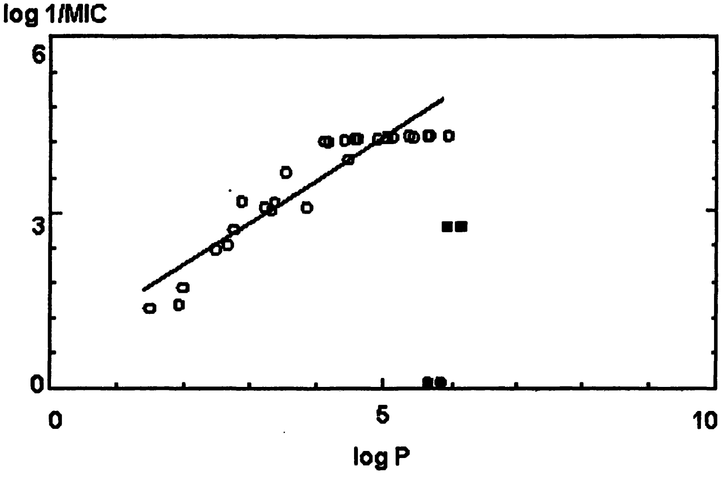

Figure 1. Dependence between MIC and logP of MOAPs Inhibiting B. cereus (Eq 1).

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Table I. Inhibitory and logP Data of MOAPs towards *B. cereus*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
2-(3-Phenylpropyl)-4-propylphenol	12.00	5.96	4.33	(*33*)
4-Isopropyl-2-(3-phenylpropyl)phenol	12.00	5.68	4.33	(*34*)
2-(3-Phenylallyl)-4-propylphenol	12.00	5.65	4.32	(*34*)
4-Ethyl-2-(3-phenylpropyl)phenol	12.00	5.43	4.30	(*34*)
4-Isopropyl-2-(3-phenylallyl)phenol	12.00	5.37	4.32	(*34*)
4-Ethyl-2-(3-phenylallyl)phenol	12.00	5.12	4.30	(*34*)
3,5-Dimethyl-2-(3-phenylallyl)phenol	12.00	5.04	4.30	(*34*)
4-Methyl-2-(3-phenylpropyl)phenol	12.00	4.90	4.28	(*34*)
2-Methyl-4-(3-phenylpropyl)phenol	12.00	4.90	4.28	(*33*)
2-Methyl-4-(3-phenylallyl)phenol	12.00	4.60	4.27	(35)
4-Methyl-2-(3-phenylallyl)phenol	12.00	4.60	4.27	(35)
4-Methyl-2-(3-phenylallyl)phenol	12.00	4.60	4.27	(34)
2-Methyl-4-(3-phenylallyl)phenol	12.00	4.60	4.27	(34)
3-Methyl-4-(3-phenylallyl)phenol	12.00	4.60	4.27	(34)
2-Methyl-6-(3-phenylallyl)phenol	12.00	4.55	4.27	(*34*)
4-(3-Phenylpropyl)phenol	25.00	4.45	3.93	(*34*)
2-(3-Phenylpropyl)phenol	12.00	4.40	4.25	(*34*)
4-(3-Phenyl-2-propenyl)phenol	12.50	4.15	4.23	(*35*)
2-(3-Phenylallyl)phenol	12.00	4.10	4.24	(*35*)
4-(2,2-Dimethylpropyl)phenol	125.00	3.83	3.12	(*36*)
2-n-Butylphenol	29.00	3.51	3.71	(*16*)
2-Methyl-5-isopropylphenol (Carvacrol)	95.00	3.35	3.20	(*37*)
4-(1,1-Dimethylethyl)phenol	125.00	3.30	3.08	(*36*)
2-Isopropyl-5-methylphenol (Thymol)	113.00	3.20	3.12	(*38*)
2-Phenylphenol (o-Phenylphenol)	100.00	2.86	3.23	(*39*)
2-(1-Propenyl)phenol (Propenylphenol)	250.00	2.73	2.73	(*36*)
1-Naphthol	500.00	2.65	2.46	(*36*)
2-Naphthol	500.00	2.65	2.46	(*36*)
3,5-Dimethylphenol	500.00	2.47	2.39	(*36*)
4-Methylphenol (para-Cresol)	2000.00	1.97	1.73	(*36*)
2-Methylphenol (ortho-Cresol)	4000.00	1.92	1.43	(*36*)
Phenol	4000.00	1.48	1.37	(*36*)
MOAPs not used in regression analysis				
Low activity (■)				
2-Isopropyl-5-methyl-4-(3-phenylpropyl)phenol	500.00	6.13	2.73	(33)
5-Isopropyl-2-methyl-4-(3-phenylallyl)phenol	500.00	5.97	2.73	(34)

Continued on next page.

Table I. *Continued.*

MOAPs not used in regression analysis	*NIC*	*logP*	*lgMIC*	*Ref*
Inactive MOAPs (●)				
2-Isopropyl-5-methyl-4-(3-phenylallyl)phenol	500.00	5.82	---	(*34*)
6-Isopropyl-3-methyl-2-(3-phenylallyl)phenol	500.00	5.62	---	(*34*)

Correlation quality: high dependence ($r^2 = 0.876$) of antimicrobial activity from lipophilicity.

$LogP_0$ definition: ~6. The activity of MOAPs increased up to a logP of 5.5. No examples are available in the range of logP of 5.5 to 6.5 (Figure 2). Data on aliphatics indicate a $logP_0$ of ~6.

Comparison with aliphatics: tetradecanol was inhibitory (MIC = 40 µg/ml, logP = 6.1, but not pentadecanol (MIC >1000 µg/ml, logP = 6.6 (*83*)).

Eq: $\log 1/MIC = 1.028 + 0.615 * \log P, n = 20, r^2 = 0.876, s = 0.244$ (2)

Specific mechanisms of action: the highly lipophilic compounds (logP = 6.7 to 8.9) are suspected to have specific activity: compounds (**2-7,11**) are related to pisferic acid (**1**), which is may be the first peptidoglycan synthesis inhibitor found among plant derived terpenoids. It impairs growth of *B. subtilis* and inhibits also growth of other gram-positive, but not gram-negative bacteria (*48*). Like anacardic acid long chain phenols, such as 3-[8(Z)-Pentadecenyl]phenol, 3-[8(Z),11(Z),14-Pentadecatrienyl]phenol, may either specifically impair membrane functions in *S. aureus* (*51*) or nonspecifically disarrange and disrupt

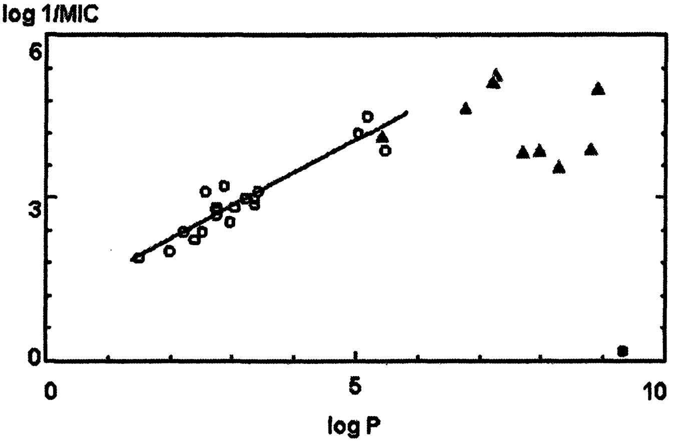

Figure 2. Dependence between MIC and logP of MOAPs Inhibiting B. subtilis (Eq 2).

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Table II. Inhibitory and logP Data of MOAPs towards *B. subtilis*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
2,6-Di-tert-butyl-4-methylphenol (BHT)	25.00	5.43	3.95	(*40*)
7-Hydroxy-3,4-dihydrocadalin (**19**)	6.25	5.16	4.54	(*41*)
7-Hydroxy-cadalin (**18**)	12.50	5.02	4.23	(42)
3-Methyl-5-(1-methylethyl)phenol (m-Thymol)	115.00	3.40	3.12	(43)
3-Methyl-4-isopropylphenol (p-Thymol)	200.00	3.35	2.88	(43)
2-Methyl-5-isopropylphenol (Carvacrol)	150.00	3.35	3.00	(43)
2-Isopropyl-5-methylphenol (Thymol)	150.00	3.20	3.00	(43)
4-Propylphenol	200.00	3.03	2.83	(4)
2-Hydroxy-6-methylallylbenzene	400.00	2.95	2.57	(4)
2-Phenylphenol (o-Phenylphenol)	100.00	2.86	3.23	(44)
2-(1-Propenyl)phenol (Propenylphenol)	200.00	2.73	2.83	(*4*)
(Z)-4-(1-Propenyl)phenol	225.00	2.73	2.78	(*4*)
(E)-4-(1-Propenyl)phenol (Anol)	275.00	2.73	2.69	(*4*)
(Z)-2-(1-Propenyl)phenol	225.00	2.73	2.78	(*4*)
4-Allylphenol (Chavicol)	100.00	2.55	3.13	(*4*)
2-Allylphenol (ortho-Chavicol)	550.00	2.50	2.39	(*4*)
2,6-Dimethylphenol (2,6-Xylenol)	700.00	2.37	2.24	(*4*)
4-Vinylphenol	500.00	2.20	2.38	(*45*)
4-Methylphenol (para-Cresol)	1000.00	1.97	2.03	(*4*)
Phenol	1150.00	1.48	1.91	(*43*)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
3-[8(Z)-Pentadecenyl]phenol	3.13	8.90	4.99	(*46*)
4b-Butylferruginol (7)	50.00	8.80	3.82	(*47*)
4b-Propylferruginol (6)	100.00	8.27	3.50	(*48*)
3-[8(Z),11(Z),14-Pentadecatrienyl]phenol	50.00	7.93	3.78	(*46*)
4b-Ethylferruginol (**5**)	50.00	7.74	3.78	(*47*)
Totarol (**11**)	1.56	7.26	5.26	(*42*)
Ferruginol (4)	2.50	7.21	5.06	(*49*)
4a-Demethylferruginol (3)	6.30	6.74	4.64	(*48*)
4b-Demethylferruginol (2)	6.30	6.74	4.64	(48)
Xanthorrhizol isomer (22)	16.00	5.38	4.14	(50)
Xanthorrhizol (21)	16.00	5.38	4.14	(50)
Inactive MOAPs (●)	*NIC*			
4b-Pentylferruginol (8)	100.00		---	(*48*)

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the cytoplasmic membrane (*52*). DNA, RNA, protein synthesis inhibition is reported by compounds (**21**,**22**) in *E. coli imp* (*50*).

3.1.3. Enterococcus faecalis

LogP coverage: data were available of MOAPs having logP values from 3 to 6.5 (Table **III**).

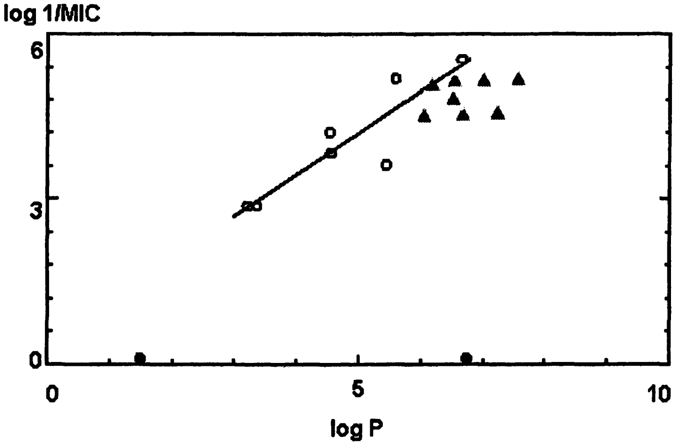

Figure 3. Dependence between MIC and logP of MOAPs Inhibiting E. faecalis (Eq 3).

Correlation quality: high dependence ($r^2 = 0.806$) of antimicrobial activity from lipophilicity.

LogP$_0$ definition: 5.5 to 6.5. A range of optima with and without benzylphenols is defined (Figure **3**).

Comparison with aliphatics: data on long chain alcohols is not available.

Eq: $\log 1/\text{MIC} = 0.441 + 0.755 * \log P$, $n = 7$, $r^2 = 0.806$, $s = 0.501$ (3)

Specific mechanisms of action: compounds (**4**,**11**-**15**) related to pisferic acid (**1**) were excluded from logP$_0$ definition. They are supposed to be inhibitors of peptidoglycan synthesis also in other bacteria then *B. subtilis* (*48*). Xibornol (**23**) causes cell membrane damage and inhibition of essential enzymes in the cell wall (*84*,*85*), which is may be true for the structurally related compound 6-exo-isocamphanyl-3,4-xylenol (**24**). Bakuchiol (**20**) inhibits DNA polymerase (*86*). In addition, the most active 2-benzylphenols (logP = 5.5 to 6.6) may have a specific mechanism of action.

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Table III. Inhibitory and logP Data of MOAPs towards *E. faecalis*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
2-Benzyl-4-hexylphenol	0.78	6.64	5.54	(*53*)
2-Benzyl-4-butylphenol	1.56	5.58	5.19	(*53*)
2,6-Di-tert-butyl-4-methylphenol (BHT)	50.00	5.43	3.64	(*40*)
3-Methyl-4-pentylphenol	25.00	4.54	3.85	(*54*)
2-Benzyl-4-ethylphenol	12.50	4.52	4.23	(*53*)
2-Methyl-5-isopropylphenol (Carvacrol)	200.00	3.35	2.88	(*55*)
2-Isopropyl-5-methylphenol (Thymol)	200.00	3.20	2.88	(55)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
14-Propylpodocarpa-8,11,13-triene-13-ol (**15**)	2.00	7.54	5.16	(*56*)
Totarol (**11**)	2.00	7.26	5.16	(*56*)
Ferruginol (**4**)	7.80	7.21	4.56	(*57*)
14-Ethylpodocarpa-8,11,13-triene-13-ol (**14**)	2.00	7.01	5.13	(*56*)
Xibornol (**23**)	3.90	6.54	4.82	(*58*)
Bakuchiol (**20**)	2.00	6.13	5.11	(*61*)
6-exo-Isocamphanyl-3,4-xylenol (**24**)	7.80	6.68	4.52	(*58*)
14-Methylpodocarpa-8,11,13-triene-13-ol (**13**)	2.00	6.49	5.11	(*56*)
Podocarpa-8,11,13-triene-13-ol (**12**)	8.00	6.04	4.48	(*56*)
Inactive MOAPs (●)	*NIC*			
Phenol	3300.00	1.48	---	(*59*)
Dihydroferruginol (**9**)	250.00	6.73	---	(*57*)

3.1.4. Listeria monocytogenes

LogP coverage: inhibitory data is available for MOAPs having logP values from 1.5 to 3.5 (Table **IV**).

Correlation quality: moderate dependence ($r^2 = 0.665$) of inhibitory activity from lipophilicity.

LogP$_0$ definition: ≥3.5 (≥5). No more exact definition can be done on the basis of MOAPs (Figure **4**).

Comparison with aliphatics: data on n-primary saturated alcohols is not available. Farnesol (3,7,11-trimethyl-2,6,10-dodecatien-1-ol, logP = 5.00, MIC = 100 μg/ml) was the most lipophilic inhibitory alcohol tested (*4*).

Eq: $\log 1/\mathrm{MIC} = 0.766 + 0.577 * \log P,\ n = 10,\ r^2 = 0.665,\ s = 0.245$ (4)

Specific mechanisms of action: ferruginol (**4**) is structurally related to pisferic acid (**1**), which impairs peptidoglycan synthesis in *B. subtilis* and may inhibit growth of other gram-positive bacteria by this mechanism. (*48*).

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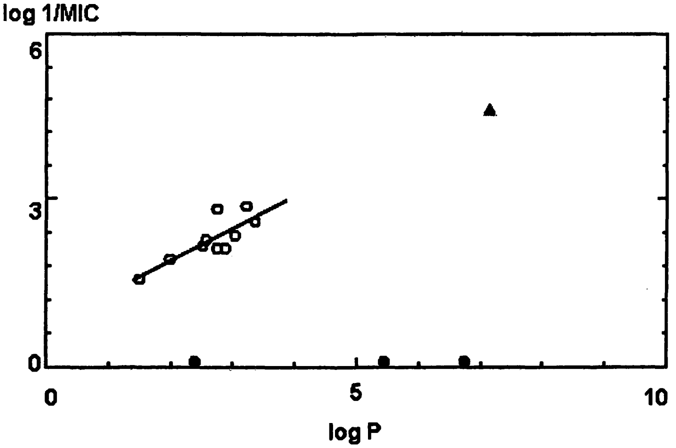

Figure 4. Dependence between MIC and logP of MOAPs Inhibiting L. monocytogenes (Eq 4).

Table IV. MIC and logP Data of MOAPs towards *L. monocytogenes*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
2-Methyl-5-isopropylphenol (Carvacrol)	380.00	3.35	2,60	(*37*)
2-Isopropyl-5-methylphenol (Thymol)	200.00	3.20	2.88	(*4*)
4-Propylphenol	600.00	3.03	2.36	(*4*)
2,4,6-Trimethylphenol (Mesitol)	1000.00	2.87	2.13	(*4*)
(Z)-4-(1-Propenyl)phenol	1000.00	2.73	2.13	(*4*)
(E)-4-(1-Propenyl)phenol (Anol)	200.00	2.73	2.83	(*4*)
4-Allylphenol (Chavicol)	700.00	2.55	2.28	(*4*)
2-Allylphenol (ortho-Chavicol)	900.00	2.50	2.17	(*4*)
4-Methylphenol (para-Cresol)	1250.00	1.97	1.94	(*4*)
Phenol	2500.00	1.48	1.58	(*4*)
MOAPs not used in regression analysis)				
Active – mechanism of action suggested (▲)				
Ferruginol (4)	7.80	7.21	4.56	(*57*)
Inactive MOAPs (●)	*NIC*			
Dihydroferruginol (9)	250.00	6.73	---	(57)
2,6-Di-tert-butyl-4-methylphenol (BHT)	512.00	5.43	---	(60)
2,6-Dimethylphenol (2,6-Xylenol)	3000.00	2.37	---	(4)

3.1.5. Micrococcus luteus

LogP coverage: data of lipophilic MOAPs having logP ≥ 6 is not available (Table **V**).

Correlation quality: moderate dependence (r^2 = 0.794) of inhibitory activity from lipophilicity.

LogP$_0$ definition: 6. Inhibitory activity of MOAPs increased up to a logP close to 6 (Figure **5**).

Comparison with aliphatics: in a series of hydroquinones substituted with aliphatic chains of increasing length the compounds 2-(3-methylbutyl)hydroquinone (MIC = 6.25 µg/ml, logP = 3.24), 2-(3,7-dimethyl-octyl)hydroquinone (MIC = 6.25 µg/ml, logP = 5.76) and 2-(3,7,11-trimethyl-dodeca-2,6,10-trienyl)benzene-1,4-diol (MIC = 12.5 µg/ml, logP = 6.82) were inhibitory towards *M. luteus*, while 2-(3,7,11-trimethyl-dodecyl)hydroquinone (logP = 8.27) and higher prenylated products were inactive (*87*). Similar to MOAPs highest activity was obtained at logP of 5.76 in this series.

Eq: $\log 1/\text{MIC} = 1.521 + 0.533 * \log P,\ n = 25,\ r^2 = 0.794,\ s = 0.281$ (5)

Specific mechanisms of action: the most active 2-benzylphenols may have a specific mechanism of action.

3.1,6. Propionibacterium acnes

LogP coverage: MOAPs cover a logP spectrum from 3 to 5.5 only (Table **VI**).

Correlation quality: high dependence (r^2 = 0.805) of inhibitory activity from lipophilicity.

LogP$_0$ definition: ≥5.5 – 7. No inhibitory data of MOAPs are available in a logP range ≥5.5, exceptionally specific inhibitors (Figure **6**). A logP$_0$ of 7 should be taken into consideration as it could be deduced from data of aliphatic alcohols.

Comparison with aliphatics: hexadecanol was active (MIC = 1.56 µg/ml, logP = 7.2, while heptadecanol was inactive (MIC > 800 µg/ml, logP = 7.7 (*25*)).

Eq: $\log 1/\text{MIC} = 0.808 + 0.693 * \log P,\ n = 15,\ r = 0.805,\ s = 0.218$ (6)

Specific mechanisms of action: totarol (**11**) may impair peptidoglycan synthesis like the structurally related compound pisferic acid (**1**) as shown with *B. subtilis* (*48*). Long chain phenols, such as 3-[8(Z)-Pentadecenyl]phenol, 3-[8(Z),11(Z),14-Pentadecatrienyl]phenol, may either specifically impair membrane functions in *S. aureus* (*51*) or nonspecifically disarrange and disrupt the cytoplasmic membrane like the related compound anacardic acid (*52*).

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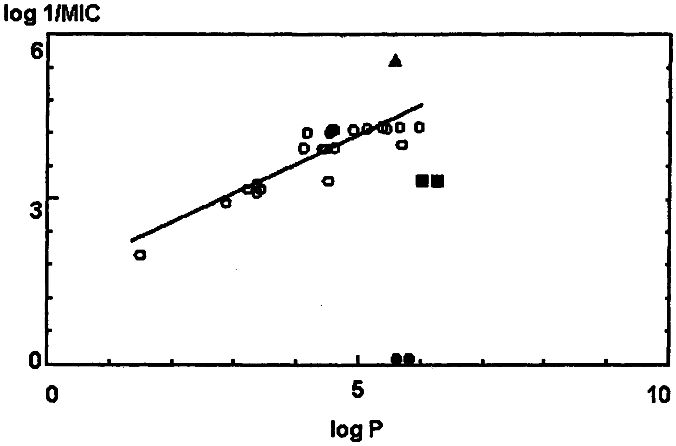

Figure 5. Dependence between MIC and logP of MOAPs Inhibiting M. luteus (Eq 5).

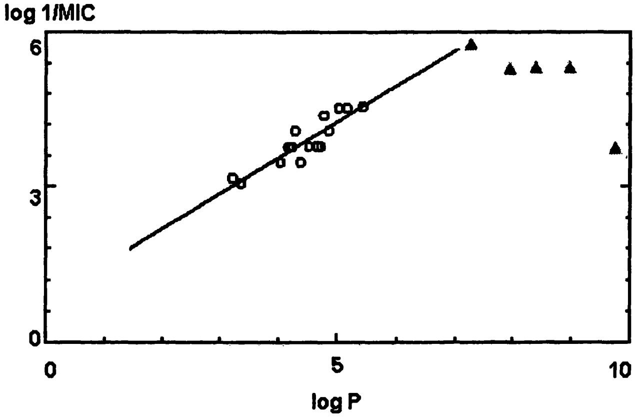

Figure 6. Dependence between MIC and logP of MOAPs Inhibiting P. acnes (Eq 6).

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Table V. Inhibitory and logP Data of MOAPs towards *M. luteus*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
2-(3-Phenylpropyl)-4-propylphenol	12.00	5.96	4.33	(*34*)
4-Isopropyl-2-(3-phenylpropyl)phenol	25.00	5.68	4.01	(*34*)
2-(3-Phenylallyl)-4-propylphenol	12.00	5.65	4.32	(*34*)
4-Ethyl-2-(3-phenylpropyl)phenol	12.00	5.43	4.30	(*34*)
4-Isopropyl-2-(3-phenylallyl)phenol	12.00	5.37	4.32	(*34*)
4-Ethyl-2-(3-phenylallyl)phenol	12.00	5.12	4.30	(*34*)
4-Methyl-2-(3-phenylpropyl)phenol	12.00	4.90	4.28	(*34*)
2-Methyl-4-(3-phenylpropyl)phenol	12.00	4.90	4.28	(33)
2-Methyl-4-(3-phenylallyl)phenol	12.00	4.60	4.27	(35)
4-Methyl-2-(3-phenylallyl)phenol	12.00	4.60	4.27	(*34*)
2-Methyl-4-(3-phenylallyl)phenol	12.00	4.60	4.27	(*34*)
3-Methyl-4-(3-phenylallyl)phenol	25.00	4.60	3.95	(*34*)
2-Methyl-6-(3-phenylallyl)phenol	12.00	4.55	4.27	(*34*)
2-Benzyl-4-ethylphenol	12.50	4.52	4.23	(*53*)
2,2,5,7,8-Pentamethylchroman-6-ol	100.00	4.50	3.34	(*62*)
4-(3-Phenylpropyl)phenol	25.00	4.45	3.93	(*34*)
2-(3-Phenylpropyl)phenol	25.00	4.40	3.93	(*34*)
4-(3-Phenyl-2-propenyl)phenol	12.50	4.15	4.23	(*35*)
2-(3-Phenylallyl)phenol	25.00	4.10	3.94	(*34*)
3-Methyl-5-(1-methylethyl)phenol (m-Thymol)	96.00	3.40	3.19	(*43*)
3-Methyl-4-isopropylphenol (p-Thymol)	77.00	3.35	3.29	(*43*)
2-Methyl-5-isopropylphenol (Carvacrol)	115.00	3.35	3.12	(*43*)
2-Isopropyl-5-methylphenol (Thymol)	96.00	3.20	3.19	(*43*)
2-Phenylphenol (o-Phenylphenol)	200.00	2.86	2.93	(*35*)
Phenol	960.00	1.48	1.99	(*43*)
MOAPs not used in regression analysis				
Active – mechanism of action unknown (▲)				
2-Benzyl-4-butylphenol	0.78	5.58	5.49	(53)
Low activity (■)				
2-Isopropyl-5-methyl-4-(3-phenylpropyl)phenol	500.00	6.13	2.73	(*33*)
5-Isopropyl-2-methyl-4-(3-phenylallyl)phenol	500.00	5.97	2.73	(*34*)
Inactive MOAPs (●)	*NIC*			
2-Isopropyl-5-methyl-4-(3-phenylallyl)phenol	500.00	5.82	---	(*34*)
6-Isopropyl-3-methyl-2-(3-phenylallyl)phenol	500.00	5.62	---	(*34*)

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Table VI. Inhibitory and logP Data of MOAPs towards *P. acnes*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
2,6-Di-tert-butyl-4-methylphenol (BHT)	6.25	5.43	4.55	(*63*)
7-Hydroxy-3,4-dihydrocadalin (**19**)	6.25	5.16	4.54	(*41*)
7-Hydroxy-cadalin (**18**)	6.25	5.02	4.54	(*42*)
5-Isopropyl-2-methyl-4-propylphenol	15.60	4.86	4.09	(*64*)
4-tert-Butyl-2-propylphenol	7.80	4.76	4.39	(*64*)
2-Isopropyl-5-methyl-4-propylphenol	31.25	4.71	3.79	(*64*)
3-Isopropyl-6-methyl-2-propylphenol	31.25	4.66	3.79	(*64*)
6-Isopropyl-3-methyl-2-propylphenol	31.25	4.51	3.79	(*64*)
4-Allyl-5-isopropyl-2-methylphenol	62.50	4.37	3.48	(64)
2-Allyl-4-tert-butylphenol	15.60	4.27	4.09	(*64*)
4-Allyl-2-isopropyl-5-methylphenol	31.25	4.22	3.78	(*64*)
2-Allyl-3-isopropyl-6-methylphenol	31.25	4.17	3.78	(*64*)
2-Allyl-6-isopropyl-3-methylphenol	62.50	4.02	3.48	(*64*)
2-Methyl-5-isopropylphenol (Carvacrol)	125.00	3.35	3.08	(*64*)
2-Isopropyl-5-methylphenol (Thymol)	100.00	3.20	3.18	(*65*)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
3-n-Pentadecylphenol (Anacardol)	50.00	9.80	3.78	(*46*)
3-[8(Z)-Pentadecenyl]phenol	1.56	8.90	5.29	(*46*)
3-[8(Z),11(Z)-Pentadecadienyl]phenol	1.56	8.41	5.28	(*46*)
3-[8(Z),11(Z),14-Pentadecatrienyl]phenol	1.56	7.93	5.28	(*46*)
Totarol (**11**)	0.39	7.26	5.87	(*42*)

3.1.7. Staphylococcus aureus

LogP coverage: the logP spectrum from 1.5 to 10 is fully covered (Table VII).

Correlation quality: high dependence (r^2 = 0.817) of inhibitory activity from lipophilicity.

LogP$_0$ definition: ~6.5. The data suggest that there is an optimum of activity at logP of ~6.5 (Figure 7).

Comparison with aliphatics: the highest activity is observed with tridecanol (20 μg/ml, logP = 5.6) and tetradecanol (40 μg/ml, logP = 6.1), while pentadecanol (logP = 7.2) was less active and hexadecanol not inhibitory (MIC = >800, logP = 7.7 (*83*)). The lipophilicity optimum of long chain aliphatic alcohols was determined to 5.1 in *S. aureus* (*52*).

Eq: $\log 1/\text{MIC} = 0.478 + 0.743 * \log P, n = 79, r^2 = 0.817, s = 0.395$ (7)

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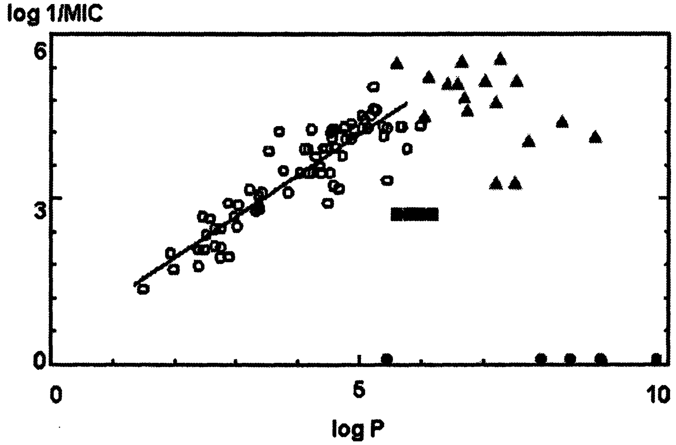

Figure 7. Dependence between MIC and logP of MOAPs Inhibiting S. aureus (Eq 7).

Specific mechanisms of action: the lipophilic (logP = 6 to 9) compounds (**2-7,11-16**) are structurally related to pisferic acid (**1**), which impairs peptidoglycan synthesis in *B. subtilis* (*48*). This may be true for other gram-positive bacteria like S. aureus. Xibornol causes (**23**) inhibition of essential enzymes (*84*), cell membrane damage and enzymatic inhibition in the cell wall (*85*), , which is may be true for the structurally related compound 6-exo-Isocamphanyl-3,4-xylenol (**24**). DNA, RNA, protein synthesis is inhibited by (**21,22**) in *E. coli imp* (*50*). Bakuchiol (**20**) inhibits DNA polymerase (*86*). In contrast to *B. subtilis* and *P. acnes* long chain phenols (logP = 8 to 10) were inactive against *S. aureus*. In addition, the most active 2-benzylphenols (logP = 5.5 to 6.6) may have a specific mechanism of action.

3.1.8. Staphylococcus epidermidis

LogP coverage: examples of MOAPs having logP values from 1.5 to 3 and 5.5 to 7 are not available (Table **VIII**).

Correlation quality: high dependence (r^2 = 0.899) of inhibitory activity from lipophilicity.

$LogP_0$ definition: ≥5.5. Failure of data limit an exact definition (Figure **8**).

Comparison with aliphatics: data on aliphatic alcohols is not available.

Eq: $\log 1/MIC = 0.940 + 0.631 * \log P,\ n = 17,\ r^2 = 0.921,\ s = 0.188$ (8)

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Table VII. Inhibitory and logP Data of MOAPs towards *S. aureus*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
2-(3-Phenylpropyl)-4-propylphenol	12.00	5.96	4.33	(*33*)
6-Hexylnaphthalen-2-ol	2.00	5.76	5.06	(*66*)
4-(6-Isopropyl-4-methyl-1-cyclohexenyl)phenol	28.00	5.76	3.92	(*67*)
4-Isopropyl-2-(3-phenylpropyl)phenol	12.00	5.68	4.33	(34)
2-(3-Phenylallyl)-4-propylphenol	12.00	5.65	4.32	(34)
4-Ethyl-2-(3-phenylpropyl)phenol	12.00	5.43	4.30	(34)
2,6-Di-tert-butyl-4-methylphenol (BHT)	100.00	5.43	3.34	(40)
Xanthorrhizol (21)	16.00	5.38	4.14	(50)
Xanthorrhizol isomer (22)	16.00	5.38	4.14	(50)
4-Isopropyl-2-(3-phenylallyl)phenol	12.00	5.37	4.32	(*34*)
6-Pentylnaphthalen-2-ol	5.00	5.26	4.63	(*66*)
7-Hydroxy-3,4-dihydrocadalin (**19**)	6.25	5.16	4.54	(*41*)
4-Ethyl-2-(3-phenylallyl)phenol	12.00	5.12	4.30	(*34*)
3,2'-Diethylbiphenyl-4-ol	7.80	5.07	4.46	(68)
3,5-Dimethyl-2-(3-phenylallyl)phenol	12.00	5.04	4.30	(34)
7-Hydroxy-cadalin (**18**)	6.25	5.02	4.54	(*41*)
2-Methyl-4-(3-phenylpropyl)phenol	12.00	4.90	4.28	(*33*)
5-Isopropyl-2-methyl-4-propylphenol	15.60	4.86	4.09	(*64*)
6-Benzylnaphthalen-2-ol	10.00	4.85	4.37	(*66*)
4-tert-Butyl-2-propylphenol	15.60	4.76	4.09	(*64*)
6-(3-Methylbutyl)naphthalene-2-ol	10.00	4.75	4.33	(*66*)
6-Butylnaphthalen-2-ol	10.00	4.74	4.30	(*66*)
2-Isopropyl-5-methyl-4-propylphenol	31.25	4.71	3.79	(*64*)
3-Isopropyl-6-methyl-2-propylphenol	125.00	4.66	3.19	(*64*)
2-Methyl-4-(3-phenylallyl)phenol	12.00	4.60	4.27	(*35*)
4-Methyl-2-(3-phenylallyl)phenol	12.00	4.60	4.27	(*35*)
4-Methyl-2-(3-phenylallyl)phenol	12.00	4.60	4.27	(*34*)
4-Methyl-2-(3-phenylpropyl)phenol	12.00	4.60	4.28	(*34*)
2-Methyl-4-(3-phenylallyl)phenol	12.00	4.60	4.27	(*34*)
3-Methyl-4-(3-phenylallyl)phenol	25.00	4.60	3.95	(*34*)
3,2'-Diethylbiphenyl-2-ol	125.00	4.57	3.26	(*68*)
2-Methyl-6-(3-phenylallyl)phenol	12.00	4.55	4.27	(*34*)
3-Methyl-4-pentylphenol	13.30	4.54	4.13	(*54*)
6-Isopropyl-3-methyl-2-propylphenol	62.50	4.51	3.49	(*64*)
2-Benzyl-4-ethylphenol	12.50	4.52	4.23	(*53*)
2-Benzyl-6-ethylphenol	250.00	4.47	2.93	(*68*)

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Table VII. ***Continued.***

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
4-(3-Phenylpropyl)phenol	25.00	4.45	3.93	(*33*)
2-(3-Phenylpropyl)phenol	25.00	4.40	3.93	(*34*)
4-Allyl-5-isopropyl-2-methylphenol	62.50	4.37	3.48	(*64*)
6-IsoButylnaphthalen-2-ol	50.00	4.35	3.60	(66)
2-Allyl-4-tert-butylphenol	31.25	4.27	3.78	(64)
4-(2,2-Dimethylpropyl)phenol	125.00	3.83	3.12	(36)
4-(1,1-Dimethylethyl)-3-methylphenol	50.00	3.75	3.52	(69)
6-Ethyl-naphthalen-2-ol	10.00	3.68	4.24	(66)
2-n-Butylphenol	20.00	3.51	3.88	(16)
3-Methyl-5-(1-methylethyl)phenol (m-Thymol)	115.00	3.40	3.12	(*43*)
(1,1'-Biphenyl)-4-ol (p-Phenylphenol)	250.00	3.36	2.83	(*68*)
3-Methyl-4-isopropylphenol (p-Thymol)	200.00	3.35	2.88	(*32*)
2-Methyl-5-isopropylphenol (Carvacrol)	125.00	3.35	3.08	(*36*)
4-(1,1-Dimethylethyl)phenol	250.00	3.30	2.78	(*36*)
2-Isopropyl-5-methylphenol (Thymol)	100.00	3.20	3.18	(*71*)
4-Propylphenol	175.00	3.03	2.89	(*4*)
2-Hydroxy-4-methylallylbenzene	450.00	3.00	2.52	(*4*)
2-Hydroxy-6-methylallylbenzene	300.00	2.95	2.69	(*4*)
2,4,6-Trimethylphenol (Mesitol)	1500.00	2.87	1.96	(*4*)
2-Phenylphenol (o-Phenylphenol)	200.00	2.86	2.93	(*70*)
2-(1-Propenyl)phenol (Propenylphenol)	250.00	2.73	2.73	(*36*)
(Z)-4-(1-Propenyl)phenol	450.00	2.73	2.47	(*4*)
(E)-4-(1-Propenyl)phenol (Anol)	1500.00	2.73	1.95	(*4*)
(Z)-2-(1-Propenyl)phenol	1000.00	2.73	2.13	(*4*)
1-Naphthol	500.00	2.65	2.46	(*36*)
2-Naphthol	1000.00	2.65	2.16	(*36*)
4-Allylphenol (Chavicol)	300.00	2.55	2.65	(*4*)
2-Allylphenol (ortho-Chavicol)	600.00	2.50	2.35	(*4*)
3,5-Dimethylphenol	1000.00	2.47	2.09	(*36*)
2-Ethylphenol (Phlorol)	250.00	2.45	2.69	(*68*)
2,6-Dimethylphenol (2,6-Xylenol)	2000.00	2.37	1.79	(*36*)
2,3-Dimethylphenol (vic-o-Xylenol)	1000.00	2.37	2.09	(*36*)
3,4-Dimethylphenol (asym.-o-Xylenol)	2000.00	2.37	1.79	(*36*)
3-Methylphenol (meta-Cresol)	2000.00	1.97	1.73	(*36*)
4-Methylphenol (para-Cresol)	2000.00	1.97	1.73	(*36*)
2-Methylphenol (ortho-Cresol)	1000.00	1.92	2.03	(*36*)
Phenol	4000.00	1.48	1.37	(*36*)

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Table VII. ***Continued.***

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
4b-Butylferruginol (**7**)	25.00	8,80	4.12	(47)
4b-Propylferruginol (**6**)	12.50	8.27	4.40	(48)
4-Allyl-2-isopropyl-5-methylphenol	62.50	4.22	3.48	(64)
6-Propyl-naphthalen-2-ol	10.00	4.21	4.27	(66)
2-Allyl-3-isopropyl-6-methylphenol	62.50	4.17	3.48	(64)
4-(3-Phenyl-2-propenyl)phenol	25.00	4.15	3.92	(35)
2-(3-Phenylallyl)phenol	25.00	4.10	3.92	(34)
2-Allyl-6-isopropyl-3-methylphenol	62.50	4.02	3.48	(*64*)
4b-Ethylferruginol (**5**)	25.00	7.74	4.08	(*47*)
14-Propylpodocarpa-8,11,13-triene-13-ol (**15**)	2.00	7.54	5.16	(*56*)
2-Isopropyl-4-(6-isopropyl-4-methyl-1-cyclohexenyl)-5-methylphenol (**17**)	140.00	7.48	3.31	(*67*)
Totarol (**11**)	0.78	7.26	5.56	(*51*)
Ferruginol (**4**)	5.00	7.21	4.76	(*49*)
Sempervirol (**16**)	150.00	7.21	3.28	(*72*)
14-Ethylpodocarpa-8,11,13-triene-13-ol (**14**)	2.00	7.01	5.13	(*56*)
4a-Demethylferruginol (**3**)	6.30	6.74	4.64	(*48*)
4b-Demethylferruginol (**2**)	6.30	6.74	4.64	(*48*)
Xibornol (**23**)	1.95	6.54	5.12	(*58*)
Bakuchiol (**20**)	1.40	6.13	5.26	(*73*)
6-exo-Isocamphanyl-3,4-xylenol (**24**)	3.90	6.68	4.82	(*58*)
14-Methylpodocarpa-8,11,13-triene-13-ol (**13**)	2.00	6.49	5.11	(*56*)
Podocarpa-8,11,13-triene-13-ol (**12**)	8.00	6.04	4.48	(*56*)
Active – mechanism of action unknown (▲)				
2-Benzyl-4-hexylphenol	0.78	6.64	5.54	(*53*)
2-Benzyl-4-butylphenol	0.78	5.58	5.49	(*53*)
Low activity (■)				
2-Isopropyl-5-methyl-4-(3-phenylpropyl)phenol	500.00	6.13	2.73	(*33*)
5-Isopropyl-2-methyl-4-(3-phenylallyl)phenol	500.00	5.97	2.73	(*34*)
2-Isopropyl-5-methyl-4-(3-phenylallyl)phenol	500.00	5.82	2.73	(*34*)
6-Isopropyl-3-methyl-2-(3-phenylallyl)phenol	500.00	5.62	2.73	(*34*)
Inactive MOAPs (●)	*NIC*	*logP*	*lgMIC*	*Ref*
3-n-Pentadecylphenol (Anacardol)	100.00	9.80	---	(*46*)
3-[8(Z)-Pentadecenyl]phenol	100.00	8.90	---	(46)
3-[8(Z),11(Z)-Pentadecadienyl]phenol	100.00	8.41	---	(46)
3-[8(Z),11(Z),14-Pentadecatrienyl]phenol	100.00	7.93	---	(*46*)
2,6-Di-tert-butyl-4-methylphenol (BHT)	100.00	5.43	---	(*40*)

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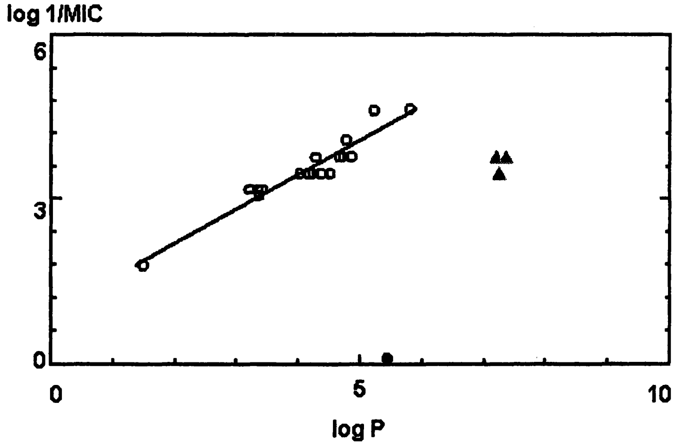

Figure 8. Dependence between MIC and logP of MOAPs Inhibiting S. epidermidis (Eq 8).

Specific mechanisms of action: totarol (**11**), ferruginol (**4**) and sempervirol (**16**) are structurally related to pisferic acid (**1**) and may inhibit peptidoglycan synthesis (*48*).

3.1.9. Streptococcus mutans

LogP coverage: data of MOAPs is available in a range of logP of 3 to 5.5 (Table **IX**).

Correlation quality: moderate dependence (r^2 = 0.621) of inhibitory activity from lipophilicity.

$LogP_0$ definition: ≥5.5. Due to the failure of data of MOAPS no more exact definition could be done (Figure 9). The below mentioned (di)-ortho-tert.-butylphenols may follow a specific mode of action and were not respected in $logP_0$ definition. The respective data on aliphatics support a $logP_0$ of 5.5.

Comparison with aliphatics: the highest activity is observed with tridecanol (MIC = 1.56 μg/ml, logP = 5.6) (*103*), while tetradecanol (logP = 6.1) was inactive (MIC >800 μg/ml, (*25*)).

Eq: $\log 1/MIC = 1.593 + 0.499 * logP, n = 12, r^2 = 0.621, s = 0.250$ (9)

Specific mechanisms of action: like anacardic acid long chain phenols, such as 3-[8(Z)-pentadecenyl]phenol, 3-[8(Z),11(Z),14-pentadecatrienyl]phenol, may either specifically impair membrane functions (*51*) or nonspecifically disarrange and disrupt the cytoplasmic membrane (*52*). Bakuchiol (**20**) inhibits DNA

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Table VIII. Inhibitory and logP Data of MOAPs towards *S. epidermidis*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
6-Hexylnaphthalen-2-ol	5.00	5.79	4.66	(*66*)
6-Pentylnaphthalen-2-ol	5.00	5.26	4.63	(*66*)
5-Isopropyl-2-methyl-4-propylphenol	31.25	4.86	3.79	(*64*)
4-tert-Butyl-2-propylphenol	15.60	4.76	4.09	(*64*)
2-Isopropyl-5-methyl-4-propylphenol	31.25	4.71	3.79	(*64*)
3-Isopropyl-6-methyl-2-propylphenol	31.25	4.66	3.79	(*64*)
6-Isopropyl-3-methyl-2-propylphenol	62.50	4.51	3.49	(*64*)
4-Allyl-5-isopropyl-2-methylphenol	62.50	4.37	3.48	(*64*)
2-Allyl-4-tert-butylphenol	31.25	4.27	3.78	(*64*)
4-Allyl-2-isopropyl-5-methylphenol	62.50	4.22	3.48	(*64*)
2-Allyl-3-isopropyl-6-methylphenol	62.50	4.17	3.48	(*64*)
2-Allyl-6-isopropyl-3-methylphenol	62.50	4.02	3.48	(*64*)
3-Methyl-5-(1-methylethyl)phenol (m-Thymol)	96.00	3.40	3.19	(*43*)
3-Methyl-4-isopropylphenol (p-Thymol)	96.00	3.35	3.19	(*43*)
2-Methyl-5-isopropylphenol (Carvacrol)	125.00	3.35	3.08	(*64*)
2-Isopropyl-5-methylphenol (Thymol)	96.00	3.20	3.19	(*43*)
Phenol	1540.00	1.48	1.79	(*43*)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
Totarol (**11**)	50.00	7.26	3.76	(*74*)
Ferruginol (**4**)	100.00	7.21	3.46	(*75*)
Sempervirol (**16**)	50.00	7.21	3.76	(*72*)
Inactive MOAPs (●)	*NIC*			
2,6-Di-tert-butyl-4-methylphenol (BHT)	100.00	5.43	---	(*40*)

polymerase (*86*). Xanthorrhizol (**21**) inhibits DNA, RNA and protein synthesis as demonstrated in *E. coli imp* (*50*). Totarol (**11**) is structurally related to pisferic acid (**1**) and may inhibit peptidoglycan synthesis (*48*). Two series of compound were excluded from regression analysis, which were very active ortho-tert.-butylphenols and less active di-ortho-tert.-butylphenols. The activity of both compounds series was not parallel with MOAPs (Figure 9).

3.2. Gram-Negative Bacteria

Structural parts of the cell envelope of gram-negative bacteria are capsule, outer membrane, peptidoglycan cell wall and cytoplasmic membrane. Capsules

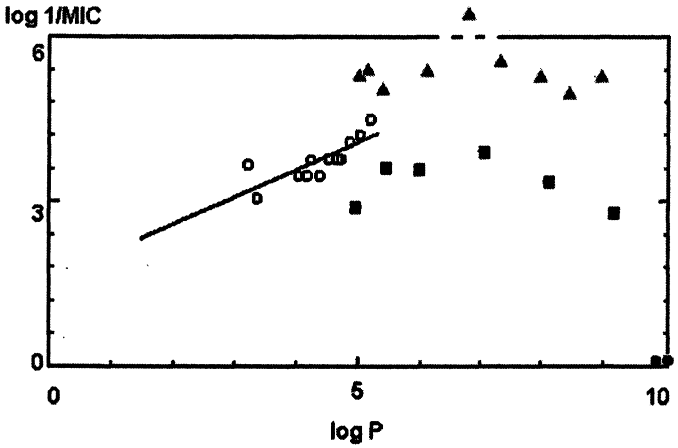

Figure 9. Dependence between MIC and logP of MOAPs Inhibiting Str. mutans (Eq 9)

are present in many species and they consist of polysaccharides (e.g. glucuronic, mannuronic acid, glucose, fucose, cellulose, glucan) and in part of lipids. The thickness of the envelope is only 10 nm, but its total mass comprises about 30% of the total dry weight of the bacterial cell mass. The envelope consists of proteins including phospholipase A1 and protease(s) (9%), lipopolysaccharides (13%), lipids and lipoproteins (>50%). The content of the peptidoglycan layer is much lower than in gram-positive bacteria (10-20%) and teichoic acids are absent. Porins enable the passage of molecules (small hydrophilic compounds, nutrients, inhibitors) up to a molecular weight of 750 daltons (*27,81*).

The outer membrane is asymmetrically built: inside phospholipids are regularly present and outside lipopolysaccharides (LPS, endotoxin). LPS is built up of a hydrophilic and a lipophilic component. The hydrophilic component consists of core oligosaccharide and O-antigen (O-specific polysaccharide), while the lipophilic component, e.g. lipid A in Enterobacteriaceae, is composed of twofold phosphorylated glucosamine disaccharide with six or seven ester or amide bond fatty acids (*27,28*). Some of the fatty acids of lipid A are hydroxylated preferably in position three and are esterified with one further fatty acid giving acyl-oxy-acyl substructures. The structures of glucosamine acyl linked fatty acids are typical for every gram-negative bacterial species (*82*).

The carbohydrate chains of the LPS may form a surface layer that acts as a barrier against the diffusion of hydrophobic molecules (*81*). The lipophilicities of this surface is calculated with the free form of fatty acids and comprises a spectrum from logP of 2.5 (3-hydroxy-decanoic acid, *Ps. aeruginosa*) to a logP of 12 (3-myristoyloxy-myristic acid, *E. coli, Sal. typhimurium*).

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Table IX. Inhibitory and logP Data of MOAPs towards *Str. mutans*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
7-Hydroxy-3,4-dihydrocadalin (**19**)	6.25	5.16	4.54	(*41*)
7-Hydroxy-cadalin (**18**)	12.50	5.02	4.23	(*41*)
5-Isopropyl-2-methyl-4-propylphenol	15.60	4.86	4.09	(*64*)
2-Isopropyl-5-methyl-4-propylphenol	31.25	4.71	3.79	(*64*)
2-Allyl-3-isopropyl-6-methylphenol	62.50	4.17	3.48	(64)
2-Allyl-6-isopropyl-3-methylphenol	62.50	4.02	3.48	(64)
2-Methyl-5-isopropylphenol (Carvacrol)	125.00	3.35	3.08	(76)
2-Isopropyl-5-methylphenol (Thymol)	31.25	3.20	3.68	(77)
2-Allyl-3-isopropyl-6-methylphenol	62.50	4.17	3.48	(64)
2-Allyl-6-isopropyl-3-methylphenol	62.50	4.02	3.48	(*64*)
2-Methyl-5-isopropylphenol (Carvacrol)	125.00	3.35	3.08	(*76*)
2-Isopropyl-5-methylphenol (Thymol)	31.25	3.20	3.68	(*77*)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
3-[8(Z)-Pentadecenyl]phenol	1.56	8.90	5.29	(*46*)
3-[8(Z),11(Z)-Pentadecadienyl]phenol	3.13	8.41	4.98	(*46*)
3-[8(Z),11(Z),14-Pentadecatrienyl]phenol	1.56	7.93	5.28	(*46*)
Totarol (**11**)	0.78	7.26	5.56	(*78*)
Bakuchiol (**20**)	1.00	6.13	5.41	(*61*)
Xanthorrhizol (**21**)	2.00	5.38	5.04	(*50*)
Active – mechanism of action unknown (▲)				
4,4'-Di-tert-butylbiphenyl-3-ol	0.12	6.92	6.36	(*77*)
4-tert-Butylbiphenyl-3-ol	0.98	5.09	5.37	(*77*)
2.4-di-t-Butylphenol	0.98	5.03	5.32	(*79*)
Low activity (■)				
2,6-Di-tert-butyl-4-octyl-phenol	500.00	9.13	2.80	(*80*)
2,6-Di-tert-butyl-4-hexylphenol	125.00	8.07	3.37	(*80*)
4-Butyl-2,6-di-tert-butylphenol	31.00	7.01	3.93	(*80*)
2,6-Di-tert-butyl-4-ethylphenol	62.00	5.96	3.58	(80)
2,6-Di-tert-butyl-4-methylphenol (BHT)	50.00	5.43	3.64	(63)
2,6 Di-tert-butylphenol	250.00	4.93	2.92	(80)
Inactive MOAPs (●)	*NIC*			
2,6-Di-tert-butyl-4-decylphenol	1000.00	10.2	---	(*80*)
3-n-Pentadecylphenol (Anacardol)	100.00	9.80	---	(*46*)

A diffusion of lipophilic compounds through porins of the outer membrane into the inner parts of the cell seems to be critical, because this process was found to be inversely dependent from hydrophobicity in a series of beta-lactam compounds in *E. coli*. Further, it seems unlikely that the same quantitative relationship exists for smaller and larger compounds (*81*).

Once a lipophilic compound has passed through the hydrophilic capsule of gram-negative bacteria, adsorption of compound material at the lipid layer located on the bacterial surface (lipid A acyl esters, logP = 2.5 to 12) may delay its further movement into the inner part of the cell in the diffusion process through the envelope. This is followed by desorption and diffusion to the outer membrane, where further compound material is inactivated due to hydrophobic interactions or it solutes in the inner part of the outer membrane and causes disorganization followed by destruction. Further passage through the small, hydrophilic peptidoglycan layer may not cause a long-lasting interruption of compound movement through the envelope. The third barrier is the cytoplasmic membrane, which differs not much (*81*) from the outer membrane in the composition of fatty acids (XlogP ~10) and in the influence of solutes on it.

Partial or total adsorption and loss of compound material may take place in all of these barriers. In consequence, a larger amount (MIC) of unspecifically acting lipophilic compounds with appropriate logP is required to cause cell death in gram-negative bacteria, if compared to gram-positive ones.

Lipophilic MOAPs of logP values >4.5 were inactive and those below were active towards gram-negative bacteria with a rare exceptions. LogP_0 (3.5 to 4.5) is almost equal to 4, the reported optimum by Hansch (*21*): *E. coli*, *Sal. typhi* 4.5., *Ps. aeruginosa* 3.5 to 4 and *K. pneumoniae* ≥3.5.

3.2.1. Escherichia coli

LogP coverage: the examined phenols comprise the lipophilicity range up to logP of 10 (Table **X**).

Correlation quality: moderate dependence ($r^2 = 0.602$) of inhibitory activity from lipophilicity.

LogP_0 definition: 4.5, MOAPs were active up to logP of 4 to 4.5 (Figure **10**).

Comparison with aliphatics: highest activity was observed with decanol (MIC = 175 µg/ml, logP = 4.0), while undecanol was less (MIC = 900 µg/ml, logP = 4.5) and higher homologues were not active (*4*).

Eq: $\log 1/\text{MIC} = 1.022 + 0.549 * \log P,\ n = 37,\ r^2 = 0.602,\ s = 0.376$ (10)

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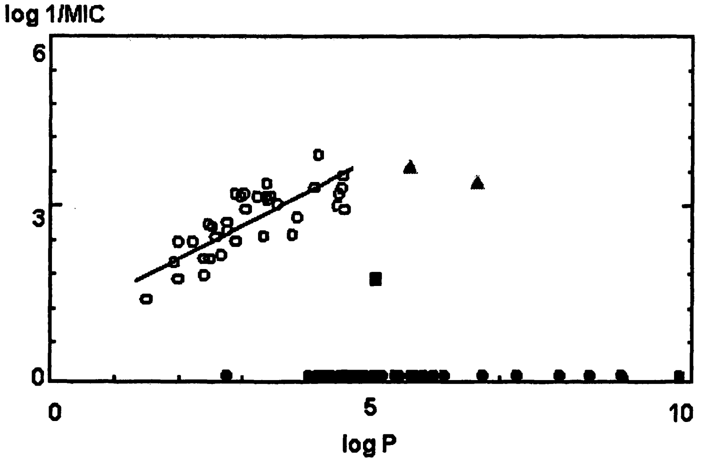

Figure 10. Dependence between MIC and logP of MOAPs Inhibiting E. coli (Eq 10).

Specific mechanisms of action: Among active compounds 2-benzylphenols (logP = 4.5 to 6.6) may have a specific mechanism of action.

3.2.2. Klebsiella pneumoniae

LogP coverage: no data is available for MOAPs in the logP range from 3.5 to 5.5 (Table **XI**).

Correlation quality: high dependence (r^2 = 0.919) of inhibitory activity from lipophilicity.

$LogP_0$ definition: ≥3.5. Due to the failure of logP data in the range between 3.5 and 5.5 the lipophilic optimum $logP_0$ isn't characterized exactly (Figure **11**).

Comparison with aliphatics: data on long chain alcohols is not available.

Eq: log 1/MIC = 0.526 + 0.776 * logP, n = 14, r = 0.919, s = 0.133 (11)

Specific mechanisms of action: DNA, RNA, protein synthesis inhibition is reported by (**21,22**) in *E. coli imp* (*50*) and peptidoglycan biosynthesis inhibition by compounds related to pisferic acid (**9-13**) (*48*). Xibornol (**19**) causes inhibition of essential enzymes (*84*), cell membrane damage and enzymatic inhibition in the cell wall (*85*), which is may be true for the structurally related compound 6-exo-Isocamphanyl-3,4-xylenol (**24**).

3.2.3. Pseudomonas aeruginosa

LogP coverage: continuously from 1.5 to 9.5 (Table **XII**).

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Table X. Inhibitory and logP Data of MOAPs towards *E. coli*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
3,2'-Diethylbiphenyl-2-ol	250.00	4.57	2.96	(*43*)
3-Methyl-4-pentylphenol	50.00	4.54	3.55	(*4*)
2-Benzyl-4-ethylphenol	100.00	4.52	3.33	(*43*)
2-Benzyl-6-ethylphenol	125.00	4.47	3.23	(*4*)
4-(3-Phenylpropyl)phenol	200.00	4.45	3.03	(*43*)
4-(3-Phenyl-2-propenyl)phenol	25.00	4.15	3.92	(*4*)
2-(3-Phenylallyl)phenol	100.00	4.10	3.34	(*88*)
4-(2,2-Dimethylpropyl)phenol	250.00	3.83	2.82	(*34*)
4-(1,1-Dimethylethyl)-3-methylphenol	500.00	3.75	2.52	(*53*)
2-n-Butylphenol	133.00	3.51	3.05	(*53*)
3-Methyl-5-(1-methylethyl)phenol (m-Thymol)	96.00	3.40	3.19	(*68*)
4-Phenylphenol (p-Phenylphenol)	125.00	3.36	3.13	(*68*)
3-Methyl-4-isopropylphenol (p-Thymol)	60.00	3.35	3.40	(*16*)
2-Methyl-5-isopropylphenol (Carvacrol)	100.00	3.35	3.18	(*4*)
4-(1,1-Dimethylethyl)phenol	500.00	3.30	2.48	(*4*)
2-Isopropyl-5-methylphenol (Thymol)	100.00	3.20	3.18	(*4*)
4-Propylphenol	150.00	3.03	2.96	(*36*)
2-Hydroxy-4-methylallylbenzene	90.00	3.00	3.22	(34)
2-Hydroxy-6-methylallylbenzene	100.00	2.95	3.17	(68)
2,4,6-Trimethylphenol (Mesitol)	546.00	2.87	2.40	(68)
2-Phenylphenol (o-Phenylphenol)	100.00	2.86	3.23	(36)
2-(1-Propenyl)phenol (Propenylphenol)	250.00	2.73	2.73	(36)
(Z)-2-(1-Propenyl)phenol	350.00	2.73	2.58	(4)
2-Naphthol	1000.00	2.65	2.16	(36)
1-Naphthol	1000.00	2.65	2.16	(36)
4-Allylphenol (Chavicol)	450.00	2.55	2.47	(4)
2-Allylphenol (ortho-Chavicol)	300.00	2.50	2.65	(4)
3,5-Dimethylphenol	1000.00	2.47	2.09	(36)
2-Ethylphenol (Phlorol)	250.00	2.45	2.69	(89)
2,6-Dimethylphenol (2,6-Xylenol)	2000.00	2.37	1.79	(36)
2,3-Dimethylphenol (vic-o-Xylenol)	1000.00	2.37	2.09	(*36*)
3,4-Dimethylphenol (asym.-o-Xylenol)	2000.00	2.37	1.79	(*36*)
4-Vinylphenol	500.00	2.20	2.38	(*90*)
3-Methylphenol (meta-Cresol)	450.00	1.97	2.38	(*4*)
4-Methylphenol (para-Cresol)	2000.00	1.97	1.73	(*36*)
2-Methylphenol (ortho-Cresol)	1000.00	1.92	2.03	(*36*)

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Table X. *Continued.*

MOAPs used in regression analysis	*NIC*	*logP*	*lgMIC*	*Ref*
Phenol	4000.00	1.48	1.37	(*36*)
MOAPs not used in regression analysis				
Active – mechanism of action unknown (▲)				
2-Benzyl-4-hexylphenol	100.00	6.64	3.43	(*35*)
2-Benzyl-4-butylphenol	50.00	5.58	3.68	(*53*)
Low activity (■)				
3,2'-Diethylbiphenyl-4-ol	4000.00	5.07	1.75	(*54*)
Inactive MOAPs (●)	*NIC*			
3-n-Pentadecylphenol (Anacardol)	100.00	9.80	---	(*46*)
3-[8(Z)-Pentadecenyl]phenol	100.00	8.90	---	(*46*)
3-[8(Z),11(Z)-Pentadecadienyl]phenol	100.00	8.41	---	(*46*)
3-[8(Z),11(Z),14-Pentadecatrienyl]phenol	100.00	7.93	---	(*46*)
Totarol (12)	100.00	7.26	---	(91)
Dihydroferruginol (**9**)	250.00	6.73	---	(57)
2-Isopropyl-5-methyl-4-(3-phenylpropyl)phenol	500.00	6.13	---	(33)
5-Isopropyl-2-methyl-4-(3-phenylallyl)phenol	500.00	5.97	---	(34)
2-(3-Phenylpropyl)-4-propylphenol	500.00	5.96	---	(34)
2-Isopropyl-5-methyl-4-(3-phenylallyl)phenol	500.00	5.82	---	(34)
4-Isopropyl-2-(3-phenylpropyl)phenol	500.00	5.68	---	(34)
2-(3-Phenylallyl)-4-propylphenol	500.00	5.65	---	(34)
6-Isopropyl-3-methyl-2-(3-phenylallyl)phenol	500.00	5.62	---	(34)
4-Ethyl-2-(3-phenylpropyl)phenol	500.00	5.43	---	(34)
2,6-Di-tert-butyl-4-methylphenol (BHT)	100.00	5.43	---	(40)
Xanthorrhizol (**21**)	128.00	5.38	---	(50)
Xanthorrhizol isomer (22)	128.00	5.38	---	(50)
4-Isopropyl-2-(3-phenylallyl)phenol	500.00	5.37	---	(34)
7-Hydroxy-3,4-dihydrocadalin (19)	100.00	5.16	---	(41)
4-Ethyl-2-(3-phenylallyl)phenol	500.00	5.12	---	(34)
3,5-Dimethyl-2-(3-phenylallyl)phenol	500.00	5.04	---	(34)
7-Hydroxy-cadalin (**18**)	100.00	5.02	---	(42)
4-Methyl-2-(3-phenylpropyl)phenol	500.00	4.90	---	(*34*)
2-Methyl-4-(3-phenylpropyl)phenol	500.00	4.90	---	(*33*)
5-Isopropyl-2-methyl-4-propylphenol	500.00	4.86	---	(*64*)
4-tert-Butyl-2-propylphenol	500.00	4.76	---	(*64*)
2-Isopropyl-5-methyl-4-propylphenol	500.00	4.71	---	(*64*)
3-Isopropyl-6-methyl-2-propylphenol	500.00	4.66	---	(*64*)
4-Methyl-2-(3-phenylallyl)phenol	500.00	4.60	---	(*34*)

Table X. *Continued.*

MOAPs not used in regression analysis	*NIC*	*logP*	*lgMIC*	*Ref*
2-Methyl-4-(3-phenylallyl)phenol	500.00	4.60	---	(*34*)
3-Methyl-4-(3-phenylallyl)phenol	500.00	4.60	---	(*34*)
4-Methyl-2-(3-phenylallyl)phenol	200.00	4.60	---	(*35*)
2-Methyl-4-(3-phenylallyl)phenol	200.00	4.60	---	(*35*)
2-Methyl-6-(3-phenylallyl)phenol	500.00	4.55	---	(34)
6-Isopropyl-3-methyl-2-propylphenol	500.00	4.51	---	(*64*)
4-Allyl-5-isopropyl-2-methylphenol	500.00	4.37	---	(*64*)
2-Allyl-4-tert-butylphenol	500.00	4.27	---	(*64*)
4-Allyl-2-isopropyl-5-methylphenol	500.00	4.22	---	(*64*)
2-Allyl-3-isopropyl-6-methylphenol	500.00	4.17	---	(*64*)
2-Allyl-6-isopropyl-3-methylphenol	500.00	4.02	---	(*64*)
(Z)-4-(1-Propenyl)phenol	3000.00	2.73	---	(*4*)
(E)-4-(1-Propenyl)phenol (Anol)	8000.00	2.73	---	(*4*)
4-Allyl-5-isopropyl-2-methylphenol	500.00	4.37	---	(*64*)
2-Allyl-4-tert-butylphenol	500.00	4.27	---	(*64*)
4-Allyl-2-isopropyl-5-methylphenol	500.00	4.22	---	(*64*)
2-Allyl-3-isopropyl-6-methylphenol	500.00	4.17	---	(*64*)
2-Allyl-6-isopropyl-3-methylphenol	500.00	4.02	---	(*64*)
(Z)-4-(1-Propenyl)phenol	3000.00	2.73	---	(*4*)
(E)-4-(1-Propenyl)phenol (Anol)	8000.00	2.73	---	(*4*)

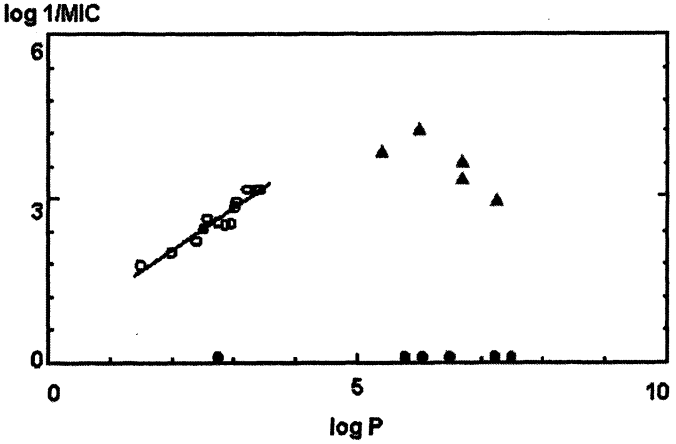

Figure 11. Dependence between MIC and logP of MOAPs Inhibiting K. pneumoniae (Eq 11).

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Correlation quality: moderate dependence (r^2 = 0.609) of inhibitory activity from lipophilicity.

$LogP_0$ definition: a $logP_0$ = 3.5 to 4 seems to be probable (Figure **12**).

Comparison with aliphatics: octanol (MIC = 2000 µg/ml, logP = 2.9) and nonanol (MIC = 160 µg/ml in presence of sodium citrate, logP = 3.6) were the highest active homologues among aliphatic alcohols, while decanol (MIC >2000 µg/ml, logP 4.0) was inactive in presence or absence of sodium citrate (*83*).

Eq: log 1/MIC = 0.742+ 0.542 * logP, n = 32, r^2 = 0.609, s = 0.368 (12)

Specific mechanisms of action: the most active 2-benzylphenols may have a specific mechanism of action. 12-demethylmulticaulin (**10**) and totarol (**11**) are structurally related to pisferic acid (**1**) and may inhibit peptidoglycan synthesis (*48*).

3.2.4. Salmonella typhi

LogP coverage: data is available from logP of 1.5 to 6 (Table **XIII**).

Correlation quality: moderate dependence (r^2 = 0.602) of inhibitory activity from lipophilicity.

$LogP_0$ definition: 4.5. This value is supported by inhibitory data of alkanols, which showed a maximum of activity at logP of 3 to 3.5, but were also active at logP of 5 (Figure **13**).

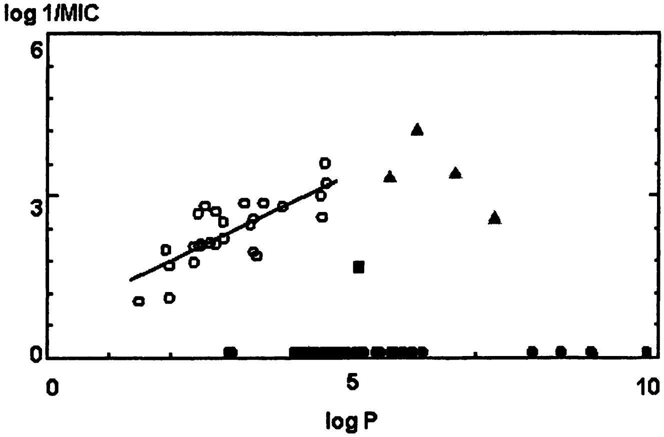

Figure 12. Dependence between MIC and logP of MOAPs Inhibiting Ps. aeruginosa (Eq 12).

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Table XI. Inhibitory and logP Data of MOAPs towards *K. pneumoniae*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
3-Methyl-5-(1-methylethyl)phenol (m-Thymol)	96.00	3.40	3.19	(*43*)
3-Methyl-4-isopropylphenol (p-Thymol)	96.00	3.35	3.19	(*43*)
2-Methyl-5-isopropylphenol (Carvacrol)	100.00	3.35	3.18	(*43*)
2-Isopropyl-5-methylphenol (Thymol)	96.00	3.20	3.19	(*43*)
4-Propylphenol	150.00	3.03	2.96	(*4*)
2-Hydroxy-4-methylallylbenzene	200.00	3.00	2.87	(*4*)
2-Hydroxy-6-methylallylbenzene	400.00	2.95	2.57	(*4*)
2-Phenylphenol (o-Phenylphenol)	500.00	2.86	2.53	(*32*)
2-(1-Propenyl)phenol (Propenylphenol)	350.00	2.73	2.58	(*4*)
4-Allylphenol (Chavicol)	300.00	2.55	2.65	(*4*)
2-Allylphenol (ortho-Chavicol)	450.00	2.50	2.47	(*4*)
2,6-Dimethylphenol (2,6-Xylenol)	700.00	2.37	2.24	(*4*)
4-Methylphenol (para-Cresol)	1000.00	1.97	2.03	(*4*)
Phenol	1500.00	1.48	1.80	(*4*)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
Totarol (**11**)	350.00	7.26	2.91	(*92*)
Xibornol (**23**)	62.50	6.68	3.62	(*58*)
6-exo-Isocamphanyl-3,4-xylenol (**24**)	125.00	6.68	3.32	(*58*)
12-Demethylmulticaulin (**10**)	15.60	6.00	4.23	(*93*)
Xanthorrhizol (21)	32.00	5.38	3.83	(*50*)
Xanthorrhizol isomer (**22**)	32.00	5.38	3.83	(*50*)
Inactive MOAPs (●)	*NIC*			
2-Isopropyl-4-(6-isopropyl-4-methyl-1-cyclo-	1000.00	7.48	----	(*67*)
Ferruginol (**4**)	32.00	7.21	----	(*56*)
14-Methylpodocarpa-8,11,13-triene-13-ol (**13**)	32.00	6.49	----	(*56*)
Podocarpa-8,11,13-triene-13-ol (**12**)	32.00	6.04	----	(*56*)
4-(6-Isopropyl-4-methyl-1-cyclohexenyl)phenol	1000.00	5.76	----	(*67*)
(Z)-4-(1-Propenyl)phenol	3000.00	2.73	----	(*4*)
(E)-4-(1-Propenyl)phenol (Anol)	3000.00	2.73	----	(*4*)

Comparison with aliphatics: highest activity is obtained with octanol (phenol coefficient (PC) = 26, logP = 2.9), while it decreases among higher homologues: nonanol (PC = 19, logP = 3.5) and dodecanol (PC = 2.75, logP = 5.0 (*95*)).

Eq: $\log 1/\mathrm{MIC} = 0.813 + 0.353 * \log P,\ n = 14,\ r^2 = 0.602,\ s = 0.283$ (13)

Table XII. Inhibitory and logP Data of MOAPs towards *Ps. aeruginosa*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
3-Methyl-4-pentylphenol	100.00	4.54	3.25	(*54*)
2-Benzyl-4-ethylphenol	50.00	4.52	3.63	(*53*)
2-Benzyl-6-ethylphenol	500.00	4.47	2.63	(*68*)
4-(3-Phenylpropyl)phenol	200.00	4.45	3.03	(*34*)
4-(2,2-Dimethylpropyl)phenol	250.00	3.83	2.82	(*36*)
2-n-Butylphenol	200.00	3.51	2.88	(16)
3-Methyl-5-(1-methylethyl)phenol (m-Thymol)	1850.00	3.40	1.91	(43)
2-Methyl-5-isopropylphenol (Carvacrol)	380.00	3.35	2.60	(94)
3-Methyl-4-isopropylphenol (p-Thymol)	1540.00	3.35	1.99	(43)
4-(1,1-Dimethylethyl)phenol	500.00	3.30	2.48	(36)
2-Isopropyl-5-methylphenol (Thymol)	200.00	3.20	2.88	(94)
2,4,6-Trimethylphenol (Mesitol)	800.00	2.87	2.23	(4)
2-Phenylphenol (o-Phenylphenol)	500.00	2.86	2.53	(70)
2-(1-Propenyl)phenol (Propenylphenol)	250.00	2.73	2.73	(36)
(Z)-2-(1-Propenyl)phenol	1000.00	2.73	2.13	(4)
2-Naphthol	1000.00	2.65	2.16	(36)
1-Naphthol	1000.00	2.65	2.16	(36)
4-Allylphenol (Chavicol)	200.00	2.55	2.83	(4)
2-Allylphenol (ortho-Chavicol)	1000.00	2.50	2.13	(*4*)
3,5-Dimethylphenol	1000.00	2.47	2.09	(*36*)
2-Ethylphenol (Phlorol)	250.00	2.45	2.69	(*68*)
2,6-Dimethylphenol (2,6-Xylenol)	2000.00	2.37	1.79	(*36*)
2,3-Dimethylphenol (vic-o-Xylenol)	1000.00	2.37	2.09	(*36*)
3,4-Dimethylphenol (asym.-o-Xylenol)	1000.00	2.37	2.09	(*36*)
4-Methylphenol (para-Cresol)	2000.00	1.97	1.73	(*36*)
3-Methylphenol (meta-Cresol)	8000.00	1.97	1.13	(*36*)
2-Methylphenol (ortho-Cresol)	1000.00	1.92	2.03	(36)
Phenol	8000.00	1.48	1.07	(36)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
Totarol (12)	750.00	7.26	2.58	(92)
12-Demethylmulticaulin (10)	15.60	6.00	4.23	(93)
Active – mechanism of action unknown (▲)				
2-Benzyl-4-hexylphenol	100.00	6.64	3.43	(53)
2-Benzyl-4-butylphenol	100.00	5.58	3.38	(53)
Low activity (■)				
3,2'-Diethylbiphenyl-4-ol	4000.00	5.07	1.75	(68)

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Table XII. *Continued.*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
Inactive MOAPs (●)	*NIC*			
3-n-Pentadecylphenol (Anacardol)	100.00	9.80	---	(46)
3-[8(Z)-Pentadecenyl]phenol	100.00	8.90	---	(46)
3-[8(Z),11(Z)-Pentadecadienyl]phenol	100.00	8.41	---	(46)
3-[8(Z),11(Z),14-Pentadecatrienyl]phenol	100.00	7.93	---	(46)
2-Isopropyl-5-methyl-4-(3-phenylpropyl)-phenol	500.00	6.13	---	(*34*)
5-Isopropyl-2-methyl-4-(3-phenylallyl)phenol	500.00	5.97	---	(*34*)
2-(3-Phenylpropyl)-4-propylphenol	500.00	5.96	---	(*34*)
2-Isopropyl-5-methyl-4-(3-phenylallyl)phenol	500.00	5.82	---	(*34*)
4-Isopropyl-2-(3-phenylpropyl)phenol	500.00	5.68	---	(*34*)
2-(3-Phenylallyl)-4-propylphenol	500.00	5.65	---	(*34*)
6-Isopropyl-3-methyl-2-(3-phenylallyl)phenol	500.00	5.62	---	(*34*)
4-Ethyl-2-(3-phenylpropyl)phenol	500.00	5.43	---	(*34*)
2,6-Di-tert-butyl-4-methylphenol (BHT)	5000.00	5.43	---	(*80*)
Xanthorrhizol (**21**)	128.00	5.38	---	(*50*)
Xanthorrhizol isomer (**22**)	128.00	5.38	---	(50)
4-Isopropyl-2-(3-phenylallyl)phenol	500.00	5.37	---	(34)
7-Hydroxy-3,4-dihydrocadalin (**19**)	800.00	5.16	---	(41)
4-Ethyl-2-(3-phenylallyl)phenol	500.00	5.12	---	(34)
3,5-Dimethyl-2-(3-phenylallyl)phenol	500.00	5.04	---	(34)
7-Hydroxy-cadalin (**18**)	100.00	5.02	---	(42)
4-Methyl-2-(3-phenylpropyl)phenol	500.00	4.90	---	(34)
2-Methyl-4-(3-phenylpropyl)phenol	500.00	4.90	---	(34)
5-Isopropyl-2-methyl-4-propylphenol	1000.00	4.86	---	(64)
4-tert-Butyl-2-propylphenol	1000.00	4.76	---	(64)
2-Isopropyl-5-methyl-4-propylphenol	1000.00	4.71	---	(64)
3-Isopropyl-6-methyl-2-propylphenol	1000.00	4.66	---	(64)
4-Methyl-2-(3-phenylallyl)phenol	500.00	4.60	---	(34)
2-Methyl-4-(3-phenylallyl)phenol	500.00	4.60	---	(34)
3-Methyl-4-(3-phenylallyl)phenol	500.00	4.60	---	(34)
3,2'-Diethylbiphenyl-2-ol	8000.00	4.57	---	(68)
2-Methyl-6-(3-phenylallyl)phenol	500.00	4.55	---	(34)
6-Isopropyl-3-methyl-2-propylphenol	1000.00	4.51	---	(64)

Continued on next page.

Table XII. *Continued.*

MOAPs not used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
2-(3-Phenylpropyl)phenol	500.00	4.40	---	(34)
4-Allyl-5-isopropyl-2-methylphenol	1000.00	4.37	---	(64)
2-Allyl-4-tert-butylphenol	1000.00	4.27	---	(64)
4-Allyl-2-isopropyl-5-methylphenol	1000.00	4.22	---	(64)
2-Allyl-3-isopropyl-6-methylphenol	1000.00	4.17	---	(64)
4-(3-Phenyl-2-propenyl)phenol	500.00	4.15	---	(*39*)
2-(3-Phenylallyl)phenol	500.00	4.10	---	(*34*)
2-Allyl-6-isopropyl-3-methylphenol	1000.00	4.02	---	(*64*)
2-Hydroxy-4-methylallylbenzene	3000.00	3.00	---	(*4*)
2-Hydroxy-6-methylallylbenzene	3000.00	2.95	---	(*4*)

Specific mechanisms of action: naphthalene derivatives (logP = >4.7) are suggested to have a specific mechanism of action.

3.3. Fungi

The fungal envelope consists regularly of plasma membrane and a cell wall built up of different layers. The inner cell wall provides mechanic stability and protects against osmotic lysis. It consists of water insoluble microfibrils and is embedded in small polysaccharides, proteins, lipids, inorganic salts, and pigments. The microfibrils are built up from chitin, a polymer of N-acetyl-D-glucosamine subunits (24 to 34.5 kDa (*98*), monomeric XlogP = -7.509).

The outer cell wall consists of polysaccharides, mainly ß-1,3-glucan (average 1.500 monomers polymerized, 240 kDa, monomeric XlogP = -7.73 (*26*)), and mannoproteins, which is a highly glycosylated polypeptide with 50 to 95% carbohydrate per weight (100 to 200 kDa). Chitin is produced in chitosomes in the cytosol. At least six different chitin synthases have been isolated from *Aspergillus* species. Activity of the enzyme ß-1,3-glucan synthase has been found in fractions of the plasma membrane and the cytosol. ß-1,6-Glucan cross-links components of the inner and outer cell wall (*99*).

In *C. albicans* the cell wall contains approx. 30 to 60% glucan, 25 to 50% mannoproteins, 1 to 2% chitin, 2 to 14% lipids and 5 to 15% proteins. A great variation of such components exists in fungi (*100,101*), e.g. the peptide trichophytin (*102*) or nigeran (polymeric-1,3- and -1,4-glucose) (*103*), glycopeptides (polymeric-1,2- and -1,6-mannose core with ß-1,4-galactose side chains, galactomannans (*104-106*)), polyphenolic pigments containing catechols

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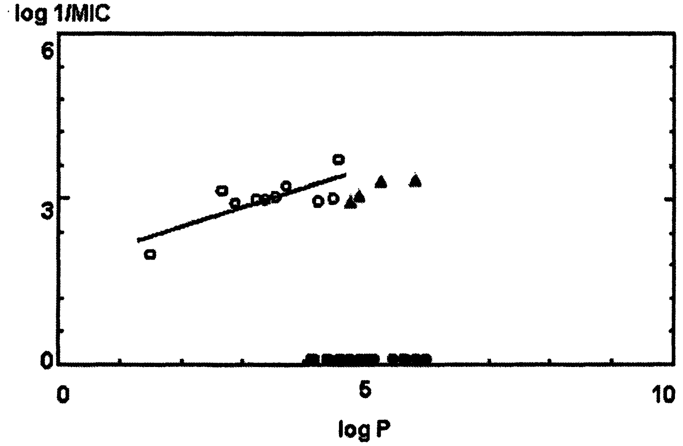

Figure 13. Dependence between MIC and logP of MOAPs Inhibiting Sal. typhi (Eq 13).

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or 1,8-dihydroxynaphthalene derived 'melanins' (*107*), chitosan (40 to 120 kDa, ß-1,4-poly-D-glucosamine: monomeric XlogP = -8.141 (*26,126*)) and further carbohydrates in cell walls of pathogenic fungi. A capsule surrounds the cell wall of *Cryptococcus neoformans*, which consists of glucuronic acid, mannose and xylose subunits (*108*).

The plasma membrane of fungi is built up from a bilayer of phospholipids, which consists of various fatty acids being typical for some species. The analysis revealed preferred occurrence of palmitoleic acid in *Candida* species, while in *Saccharomyces* species palmitoleic and oleic acid (logP 6.7 and 7.8) were most abundant fatty acids. Lauric and palmitic acid in *C. albicans* were found to be the main fatty acids (logP = 5 to 6.1) in another study. In *Trichophyton rubrum* palmitic, stearic, oleic, and in part large amounts of linoleic acid (logP = 6.3) present the major fatty acids (*109*).

The fungal plasma membrane contains typically ergosterol ((22E)-ergosta-5,7,22-trien-3beta-ol: logP = 9.0) in amounts of 0.4 to 42 µg/mg mycelial dry mass (*110*) and sphingolipids (XlogP = 7.428 (*26*)), which both influence fungal susceptibility towards antifungal drugs (*111*).

When a lipophilic compound diffuses through the cell wall consisting of polysaccharide materials (ß-1,3- or ß-1,6-glucan, chitin, chitosan, XlogP ~ -8) adsorption at these hydrophilic layers seems to be of minor importance. An unanswered question is the maximum molecule size to be permitted to diffuse through the network of microfibrils and other polysaccharides. Cell wall lipids (fatty acids: logP = 5 to 8, sphingolipids: XlogP = 7.428, ergosterol: logP = 9.0) and pigments were appropriate for hydrophobic interactions with a diffusing

Table XIII. Inhibitory and logP Data of MOAPs towards *Sal. typhi*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
3-Methyl-4-pentylphenol	33.00	4.54	3.73	(*54*)
4-(3-Phenylpropyl)phenol	200.00	4.45	3.03	(*34*)
6-Propyl-naphthalen-2-ol	200.00	4.21	2.97	(*66*)
6-Ethyl-naphthalen-2-ol	100.00	3.68	3.24	(*66*)
2-n-Butylphenol	133.00	3.51	3.05	(*16*)
2-Methyl-5-isopropylphenol (Carvacrol)	150.00	3.35	3.00	(*96*)
2-Isopropyl-5-methylphenol (Thymol)	150.00	3.20	3.00	(*96*)
2-Phenylphenol (o-Phenylphenol)	200.00	2.86	2.93	(*39*)
2-Naphthol	100.00	2.65	3.16	(*66*)
Phenol	925.00	1.48	2.01	(*97*)
MOAPs not used in regression analysis				
Active – mechanism of action unknown (▲)				
6-Hexylnaphthalen-2-ol	100.00	5.76	3.36	(*66*)
6-Pentylnaphthalen-2-ol	100.00	5.21	3.33	(*66*)
6-Benzylnaphthalen-2-ol	200.00	4.85	3.07	(*66*)
6-Butylnaphthalen-2-ol	200.00	4.74	3.00	(*66*)
Inactive MOAPs (•)	*NIC*			
5-Isopropyl-2-methyl-4-(3-phenylallyl)phenol	500.00	5.97	---	(*34*)
2-(3-Phenylpropyl)-4-propylphenol	500.00	5.96	---	(*34*)
2-Isopropyl-5-methyl-4-(3-phenylallyl)phenol	500.00	5.82	---	(*34*)
2-(3-Phenylallyl)-4-propylphenol	500.00	5.65	---	(*34*)
6-Isopropyl-3-methyl-2-(3-phenylallyl)phenol	500.00	5.62	---	(*34*)
4-Ethyl-2-(3-phenylpropyl)phenol	500.00	5.43	---	(*34*)
2,6-Di-tert-butyl-4-methylphenol (BHT)	100.00	5.43	---	(*40*)
4-Ethyl-2-(3-phenylallyl)phenol	500.00	5.12	---	(*34*)
3,5-Dimethyl-2-(3-phenylallyl)phenol	500.00	5.04	---	(34)
4-Methyl-2-(3-phenylpropyl)phenol	500.00	4.90	---	(*34*)
2-Methyl-4-(3-phenylpropyl)phenol	500.00	4.90	---	(*34*)
6-(3-Methylbutyl)naphthalen-2-ol	200.00	4.75	---	(*66*)
4-Methyl-2-(3-phenylallyl)phenol	500.00	4.60	---	(*34*)
2-Methyl-4-(3-phenylallyl)phenol	500.00	4.60	---	(*34*)
3-Methyl-4-(3-phenylallyl)phenol	500.00	4.60	---	(*34*)
2-Methyl-6-(3-phenylallyl)phenol	500.00	4.55	---	(*34*)
2-(3-Phenylpropyl)phenol	500.00	4.40	---	(*34*)
6-IsoButylnaphthalen-2-ol	200.00	4.35	---	(*66*)
4-(3-Phenyl-2-propenyl)phenol	500.00	4.15	---	(*34*)
2-(3-Phenylallyl)phenol	500.00	4.10	---	(*34*)

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compound. The nature of such interactions may cause species specific influences on the diffusion process.

MOAPs were inhibitory towards all fungi selected. Their lipophilic optimum is approx. 5 in *A. niger, T. mentagrophytes* and *C. albicans*, which points to an unspecific solubilisation in the inner part of the plasma membrane in each of these organisms.

3.3.1. Aspergillus niger

LogP coverage: the logP-spectrum is covered up to a logP of 6 (Table **XIV**).

Correlation quality: moderate dependence ($r^2 = 0.570$) of inhibitory activity from lipophilicity.

$LogP_0$ definition: 4.5. Activity increases up to a logP of 4.5 to 5 (Figure **14**), which is similarly obtained with alkanols .

Comparison with aliphatics: highest activity was observed with undecanol (MIC = 80 µg/ml, logP = 4.5), while dodecanol (MIC >2000 µg/ml, logP = 5.0) and higher homologues were not active (*83*).

Eq: $\log 1/\mathrm{MIC} = 2.440 + 0.212 * \log P$, $n = 25$, $r^2 = 0.570$, $s = 0.198$ (14)

Specific mechanisms of action: phenyl substituted phenols are suggested to have a specific mechanism of action.

3.3.2. Trichophyton mentagrophytes

LogP coverage: the spectrum is covered up to a logP of 6 (Table **XV**).

Correlation quality: high dependence ($r^2 = 0.787$) of inhibitory activity from lipophilicity.

$LogP_0$ definition: 5. This optimum is indicated by data obtained with MOAPs and alkanols (Figure **15**), however, more lipophilic phenols are untested.

Comparison with aliphatics: highest activity was observed with dodecanol (MIC = 3.13 µg/ml, logP = 5.0), while tridecanol (MIC = 800 µg/ml, logP = 5.6) was less and higher homologues were not active (*25*).

Eq: $\log 1/\mathrm{MIC} = 1.444 + 0.647 * \log P$, $r^2 = 0.787$ $n = 23$, $s = 0.361$ (15)

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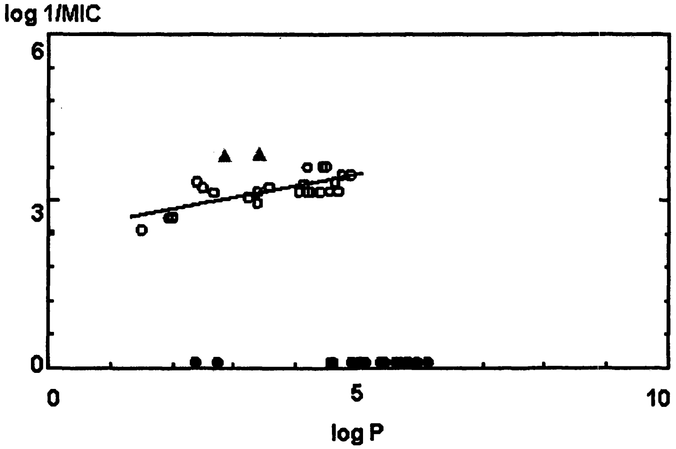

Figure 14. Dependence between MIC and logP of MOAPs Inhibiting A. niger (Eq 14).

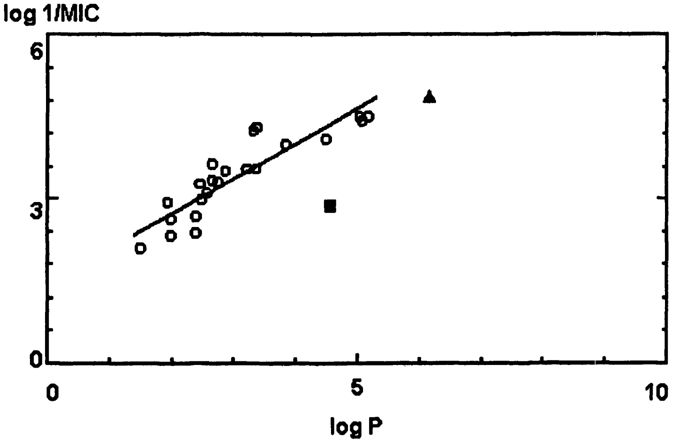

Figure 15. Dependence between MIC and logP of MOAPs Inhibiting T. mentagrophytes (Eq 15).

Table XIV. Inhibitory and logP Data of MOAPs towards *A. niger*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
5-Isopropyl-2-methyl-4-propylphenol	62.50	4.86	3.49	(*64*)
2-Isopropyl-5-methyl-4-propylphenol	62.50	4.71	3.49	(*64*)
3-Isopropyl-6-methyl-2-propylphenol	125.00	4.66	3.19	(*64*)
3-Methyl-4-(3-phenylallyl)phenol	100.00	4.60	3.35	(*34*)
6-Isopropyl-3-methyl-2-propylphenol	125.00	4.51	3.19	(*64*)
4-(3-Phenylpropyl)phenol	50.00	4.45	3.63	(*33*)
2-(3-Phenylpropyl)phenol	50.00	4.40	3.63	(*33*)
4-Allyl-5-isopropyl-2-methylphenol	125.00	4.37	3.18	(*64*)
4-Allyl-2-isopropyl-5-methylphenol	125.00	4.22	3.18	(*64*)
2-Allyl-3-isopropyl-6-methylphenol	125.00	4.17	3.18	(*64*)
4-(3-Phenyl-2-propenyl)phenol	50.00	4.15	3.62	(*34*)
2-(3-Phenylallyl)phenol	100.00	4.10	3.32	(*34*)
2-Allyl-6-isopropyl-3-methylphenol	125.00	4.02	3.18	(*64*)
4-Benzylphenol	100.00	3.54	3.27	(*112*)
2-Methyl-5-isopropylphenol (Carvacrol)	100.00	3.35	3.18	(*32*)
2-Isopropyl-5-methylphenol (Thymol)	160.00	3.35	2.97	(*113*)
4-Isopropylphenol	125.00	3.20	3.08	(*64*)
1-Naphthol	100.00	2.65	3.16	(*114*)
2-Naphthol	100.00	2.65	3.16	(*114*)
3,5-Dimethylphenol	67.00	2.47	3.26	(*115*)
2,6-Dimethylphenol (2,6-Xylenol)	55.00	2.37	3.35	(*115*)
3-Methylphenol (meta-Cresol)	215.00	1.97	2.70	(*115*)
4-Methylphenol (para-Cresol)	200.00	1.97	2.73	(*115*)
2-Methylphenol (ortho-Cresol)	215.00	1.92	2.70	(*115*)
Phenol	310.00	1.48	2.48	(*116*)
MOAPs not used in regression analysis				
Active – mechanism of action unknown (▲)				
4-Phenylphenol (p-Phenylphenol)	25.00	3.36	3.83	(*112*)
2-Phenylphenol (o-Phenylphenol)	25.00	2.86	3.83	(*44*)
Inactive MOAPs (●)	*NIC*			
2-Isopropyl-5-methyl-4-(3-phenylpropyl)phenol	500.00	6.13	---	(*33*)
2-(3-Phenylpropyl)-4-propylphenol	500.00	5.96	---	(*33*)
2-Isopropyl-5-methyl-4-(3-phenylallyl)phenol	500.00	5.82	---	(*34*)
4-Isopropyl-2-(3-phenylpropyl)phenol	200.00	5.68	---	(*34*)
2-(3-Phenylallyl)-4-propylphenol	200.00	5.65	---	(*34*)
6-Isopropyl-3-methyl-2-(3-phenylallyl)phenol	500.00	5.62	---	(*34*)
4-Ethyl-2-(3-phenylpropyl)phenol	500.00	5.43	---	(*34*)

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Table XIV. *Continued.*

MOAPs not used in regression analysis	*NIC*	*logP*	*lgMIC*	*Ref*
4-Ethyl-2-(3-phenylpropyl)phenol	500.00	5.43	---	(*34*)
4-Isopropyl-2-(3-phenylallyl)phenol	500.00	5.37	---	(*34*)
4-Ethyl-2-(3-phenylallyl)phenol	200.00	5.12	---	(*34*)
3,5-Dimethyl-2-(3-phenylallyl)phenol	500.00	5.04	---	(*34*)
4-Methyl-2-(3-phenylpropyl)phenol	200.00	4.90	---	(*34*)
2-Methyl-4-(3-phenylpropyl)phenol	200.00	4.90	---	(*33*)
2-Methyl-4-(3-phenylpropyl)phenol	200.00	4.90	---	(*33*)
4-Methyl-2-(3-phenylallyl)phenol	200.00	4.60	---	(*34*)
2-Methyl-4-(3-phenylallyl)phenol	200.00	4.60	---	(*34*)
4-Methyl-2-(3-phenylallyl)phenol	200.00	4.60	---	(*35*)
2-Methyl-4-(3-phenylallyl)phenol	200.00	4.60	---	(*35*)
2-Methyl-6-(3-phenylallyl)phenol	200.00	4.55	---	(*34*)
(Z)-2-(1-Propenyl)phenol	1000.00	2.73	---	(*4*)
2,4-Dimethylphenol (asym.-m-Xylenol)	200.00	2.37	---	(*114*)

Specific mechanisms of action: The most active compound bakuchiol (**20**) was identified as DNA polymerase inhibitors (*83*) and was therefore excluded in the definition of $\log P_0$.

3.3.3. *Candida albicans*

LogP coverage: the spectrum is covered up to a logP of 7 (Table **XVI**).

Correlation quality: moderate dependence (r^2 = 0.562) of inhibitory activity from lipophilicity.

LogP₀ definition: 5. The optimum at a logP of 5 is confirmed by inhibitory activity of alkanols (Figure **16**).

Comparison with aliphatics: highest activity was observed with dodecanol (MIC = 25 µg/ml (*124*), logP = 5.0), while the homologues undecanol (MIC = 100 µg/ml (*125*), logP = 4.5) and tridecanol (MIC = 10000 µg/ml (*126*), logP = 5.6) were less active. However, contradictory reports exist on dodecanol, which was inactive in another study (MIC >1000 µg/ml (*69*)).

Eq: $\log 1/\mathrm{MIC} = 1.740 + 0.379 * \log P,\ n = 35,\ r^2 = 0.562,\ s = 0.328$ (16)

Specific mechanisms of action: compounds (**21,22**) might inhibit DNA, RNA, and protein synthesis as shown with E. coli imp (*50*).

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Table XV. Inhibitory and logP Data of MOAPs towards *T. mentagrophytes*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
7-Hydroxy-3,4-dihydrocadalin (**19**)	6.25	5.16	4.54	(*41*)
3,2'-Diethylbiphenyl-4-ol	7.80	5.07	4.46	(*117*)
7-Hydroxy-cadalin (**18**)	6.25	5.02	4.54	(*41*)
2-Benzyl-6-ethylphenol	15.60	4.47	4.13	(*117*)
4-(2,2-Dimethylpropyl)phenol	15.60	3.83	4.02	(*36*)
4-Phenylphenol (p-Phenylphenol)	7.80	3.36	4.34	(*117*)
2-Methyl-5-isopropylphenol (Carvacrol)	40.00	3.35	3.57	(*113*)
4-(1,1-Dimethylethyl)phenol	7.80	3.30	4.28	(*36*)
2-Isopropyl-5-methylphenol (Thymol)	40.00	3.20	3.57	(*118*)
2-Phenylphenol (o-Phenylphenol)	50.00	2.86	3.53	(*119*)
2-(1-Propenyl)phenol (Propenylphenol)	62.50	2.73	3.33	(*36*)
1-Naphthol	31.25	2.65	3.66	(*36*)
2-Naphthol	62.50	2.65	3.36	(*36*)
4-Allylphenol (Chavicol)	100.00	2.55	3.13	(*120*)
3,5-Dimethylphenol	125.00	2.47	2.99	(*36*)
2-Ethylphenol (Phlorol)	62.50	2.45	3.29	(*117*)
2,3-Dimethylphenol (vic-o-Xylenol)	250.00	2.37	2.69	(*36*)
3,4-Dimethylphenol (asym.-o-Xylenol)	500.00	2.37	2.39	(*36*)
2,6-Dimethylphenol (2,6-Xylenol)	500.00	2.37	2.39	(*36*)
3-Methylphenol (meta-Cresol)	500.00	1.97	2.34	(*36*)
4-Methylphenol (para-Cresol)	250.00	1.97	2.64	(*36*)
2-Methylphenol (ortho-Cresol)	125.00	1.92	2.94	(*36*)
Phenol	750.00	1.48	2.10	(*36*)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				
Bakuchiol (**20**)	4.00	6.13	4.81	(*121*)
Low activity (■)	*NIC*			
3,2'-Diethylbiphenyl-2-ol	250.00	4.57	2.96	(*117*)

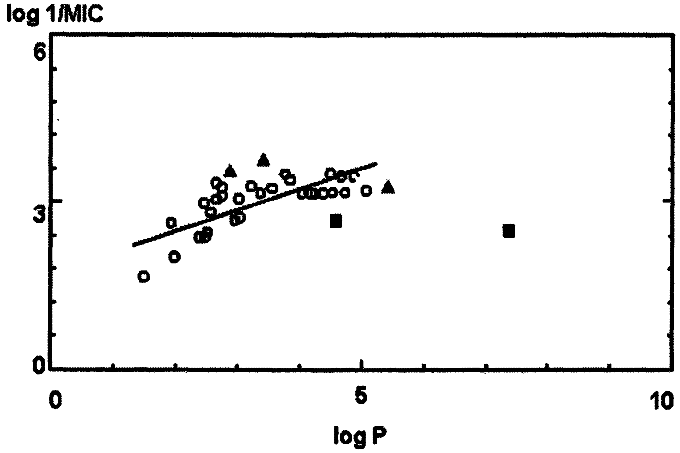

Figure 16. Dependence between MIC and logP of MOAPs Inhibiting C. albicans (Eq 16).

4. Discussion

Despite the fact that the analyzed antimicrobial inhibitory data have been established due to various testing protocols, individuals of the group of MOAPs were active against each of the 16 microbial species studied. The inhibitory activity of MOAPs increased significantly with lipophilicity, a fact already reported in common for unspecifically acting antimicrobials (*19*).

Computational calculation of lipophilicity of different compounds and correlation analysis with microbiological data confirmed the results obtained by Lien and Hansch (*21,115*), who reported the lipophilicity optima for neutral drugs acting on gram-positive ($logP_0 = 5.7 \pm 0.5$), gram-negative bacteria ($logP_0 = 4.4 \pm 0.4$) and fungi ($logP_0 = 5.6 \pm 1$). In this examination the average $logP_0$ is 5.8 ±0.7 for nine gram-positive bacteria, $logP_0 = 4.1 \pm 0.6$ for four gram-negative bacteria and $logP_0 = 5.0$ for three fungal species. Neutral acting antimicrobials with a logP >4.5 were inactive against most of gram-negative bacteria studied.

The best correlation coefficients were obtained with gram-positive bacteria (average $r^2 = 0.80$) and were lower for gram-negative bacteria (average $r^2 = 0.68$) and fungi (average $r^2 = 0.64$) in linear regression analysis (Table XVII). These differences can be explained with the different diffusion behavior of lipophilic compound through the microbial envelopes. In gram-positive bacteria an active, lipophilic compound is allowed to diffuse without much hindrance through the murein network. It enters the cytoplasmic membrane and causes damage in relation to its lipophilicity.

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Table XVI. Inhibitory and logP Data of MOAPs towards *C. albicans*

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
3,2'-Diethylbiphenyl-4-ol	125.00	5.07	3.26	(*117*)
5-Isopropyl-2-methyl-4-propylphenol	62.50	4.86	3.49	(*64*)
2-Isopropyl-5-methyl-4-propylphenol	125.00	4.71	3.19	(*64*)
3-Isopropyl-6-methyl-2-propylphenol	62.50	4.66	3.49	(*64*)
6-Isopropyl-3-methyl-2-propylphenol	125.00	4.51	3.19	(*64*)
2-Benzyl-6-ethylphenol	62.50	4.47	3.53	(*117*)
4-Allyl-5-isopropyl-2-methylphenol	125.00	4.37	3.18	(*64*)
4-Allyl-2-isopropyl-5-methylphenol	125.00	4.22	3.18	(*64*)
2-Allyl-3-isopropyl-6-methylphenol	125.00	4.17	3.18	(*64*)
2-Allyl-6-isopropyl-3-methylphenol	125.00	4.02	3.18	(*64*)
4-(2,2-Dimethylpropyl)phenol	62.50	3.83	3.42	(*36*)
4-(1,1-Dimethylethyl)-3-methylphenol	50.00	3.75	3.52	(*69*)
4-Benzylphenol	100.00	3.54	3.27	(*112*)
2-Methyl-5-isopropylphenol (Carvacrol)	100.00	3.35	3.18	(*122*)
3-Methyl-4-isopropylphenol (p-Thymol)	100.00	3.35	3.18	(*43*)
4-(1,1-Dimethylethyl)phenol	31.25	3.30	3.68	(*36*)
2-Isopropyl-5-methylphenol (Thymol)	75.00	3.20	3.30	(*123*)
4-Propylphenol	250.00	3.03	2.74	(*4*)
2-Hydroxy-4-methylallylbenzene	125.00	3.00	3.07	(*4*)
2-Hydroxy-6-methylallylbenzene	300.00	2.95	2.69	(*4*)
2-(1-Propenyl)phenol (Propenylphenol)	70.00	2.73	3.28	(*4*)
(Z)-4-(1-Propenyl)phenol	100.00	2.73	3.13	(*4*)
1-Naphthol	62.50	2.65	3.36	(*36*)
2-Naphthol	125.00	2.65	3.06	(*36*)
4-Allylphenol (Chavicol)	200.00	2.55	2.83	(*120*)
2-Allylphenol (ortho-Chavicol)	450.00	2.50	2.47	(*4*)
3,5-Dimethylphenol	500.00	2.47	2.39	(*36*)
2-Ethylphenol (Phlorol)	125.00	2.45	2.99	(*117*)
2,3-Dimethylphenol (vic-o-Xylenol)	500.00	2.37	2.39	(*36*)
2,6-Dimethylphenol (2,6-Xylenol)	500.00	2.37	2.39	(*36*)
3,4-Dimethylphenol (asym.-o-Xylenol)	500.00	2.37	2.39	(*36*)
3-Methylphenol (meta-Cresol)	1000.00	1.97	2.03	(*36*)
4-Methylphenol (para-Cresol)	1000.00	1.97	2.03	(*36*)
2-Methylphenol (ortho-Cresol)	250.00	1.92	2.64	(*36*)
Phenol	2000.00	1.48	1.67	(*36*)
MOAPs not used in regression analysis				
Active – mechanism of action suggested (▲)				

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Table XVI. ***Conitnued.***

MOAPs used in regression analysis (○)	*MIC*	*logP*	*lgMIC*	*Ref*
Xanthorrhizol (**21**)	128.00	5.38	3.23	(*50*)
Xanthorrhizol isomer (**22**)	128.00	5.38	3.23	(*50*)
4-Phenylphenol (p-Phenylphenol)	31.25	3.36	3.74	(*112*)
2-Phenylphenol (o-Phenylphenol)	50.00	2.86	3.53	(*32*)
Low activity (■)				
Totarol (**11**)	750.00	7.26	2.58	(*92*)
3,2'-Diethylbiphenyl-2-ol	500.00	4.57	2.66	(*117*)
Inactive MOAPs (●)	*NIC*			
Ferruginol (**4**)	250.00	7.21	---	(*57*)
Dihydroferruginol (**9**)	250.00	6.73	---	(*57*)
2,6-Di-tert-butyl-4-methylphenol (BHT)	5000.00	5.43	---	(*40*)
(E)-4-(1-Propenyl)phenol (Anol)	3000.00	2.73	---	(*4*)
(Z)-2-(1-Propenyl)phenol	1000.00	2.73	---	(*4*)
2,4-Dimethylphenol (asym.-m-Xylenol)	200.00	2.37	---	(*114*)

Table XVII. Statistical Data on the Dependence of Antimicrobial Activity from Lipophilicity of MOAPs

Species	*n*	*ni*	*Y*	*X*	r^2	*s*	$logP_{max.}$	$LogP_0$
SE	17	1	0.940	0.631	0.921	0.188	7.26	≥5.5
PK	14	7	0.526	0.776	0.919	0.133	7.26	≥3.5
BS	20	3	1.028	0.615	0.876	0.244	8.90	~6
BC	32	2	0.671	0.728	0.869	0.346	6.13	~6
SA	79	5	0.478	0.743	0.817	0.395	8.27	~6.5
EF	7	2	0.441	0.755	0.806	0.501	7.26	5.5 – 6.5
PA	15	0	0.808	0.693	0.805	0.218	9.80	≥5.5 (7)
ML	25	2	1.521	0.533	0.794	0.281	6.13	6
TM	23	0	1.444	0.647	0.787	0.361	6.13	5
LM	10	3	0.766	0.577	0.665	0.245	7.21	≥3.5 (≥5)
SM	12	2	1.593	0.499	0.621	0.250	9.13	≥5.5
PsA	32	43	0.742	0.542	0.609	0.368	7.26	3.5 - 4
ST	14	20	0.813	0.353	0.602	0.283	5.76	4.5
EC	37	42	1.022	0.549	0.602	0.376	6.64	4.5
AN	25	21	2.440	0.212	0.570	0.198	4.86	5
CA	35	6	1.740	0.379	0.562	0.328	7.26	5

n = number of compounds used for regression analysis, ni = number of inactive compounds, r = correlation coefficient, X = slope Y = Y Intercept, $logP_{max.}$= maximum logP reported of inhibitory MOAP, BC = *B. cereus*, BS = *B. subtilis*, EF = *E. faecalis*, LM = *L. monocytogenes*, ML = *M. luteus*, PA = *P. acnes*, SA = *Staphylococcus aureus*, SE = *S. epidermidis*, SM = *S. mutans*, EC = *Escherichia coli*, KP = *Klebsiella pneumoniae*, PsA = *P. aeruginosa*, ST = *S. typhi*, AN = *A. niger*, TM = *T. mentagrophytes*, CA = *C. albicans*.

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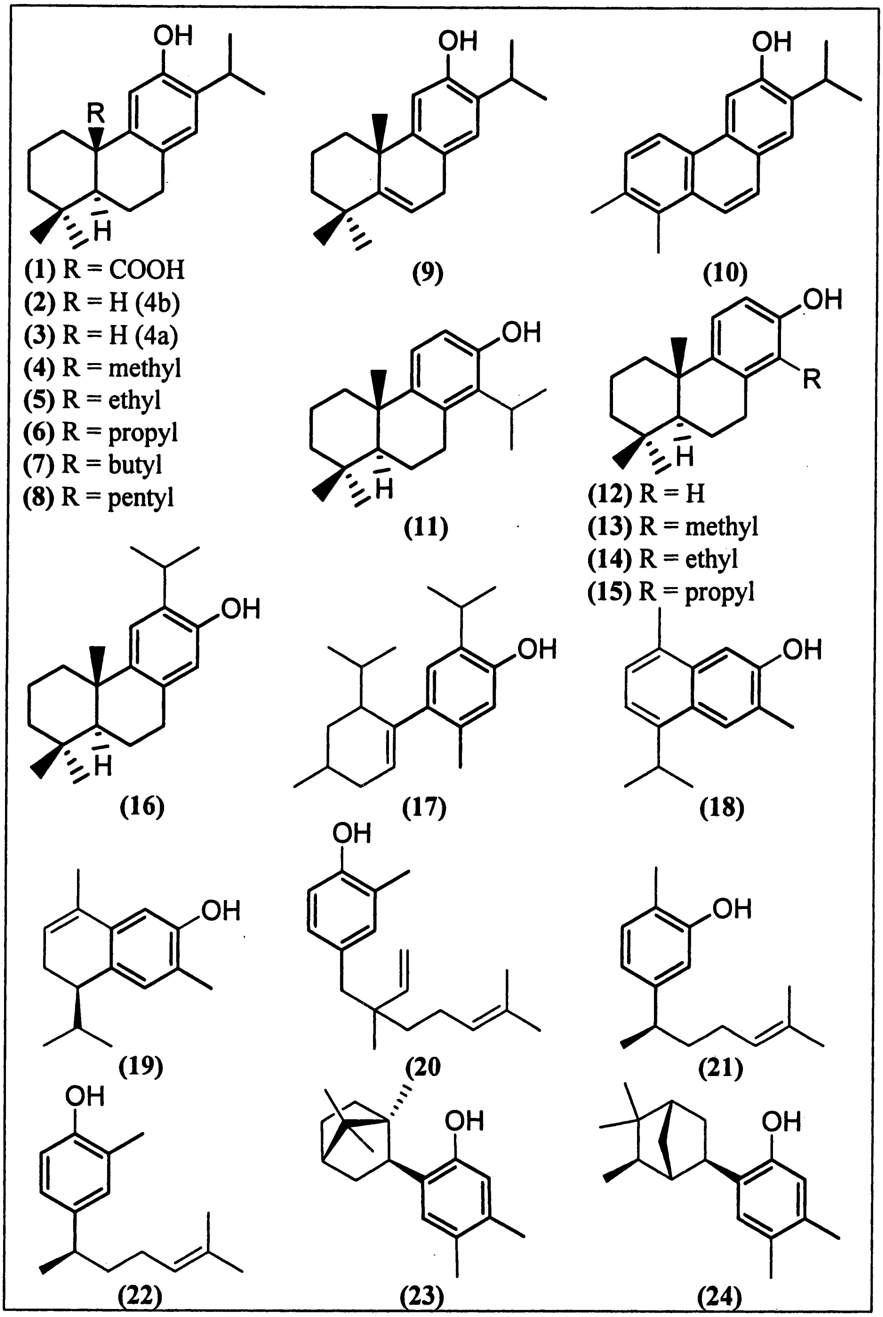
OH
R
H
(1) R = COOH
(2) R = H (4b)
(3) R = H (4a)
(4) R = methyl
(5) R = ethyl
(6) R = propyl
(7) R = butyl
(8) R = pentyl
OH
(9)
OH
(10)
OH
H
(11)
OH
R
H
(12) R = H
(13) R = methyl
(14) R = ethyl
(15) R = propyl
OH
H
(16)
OH
(17)
OH
(18)
OH
(19)
OH
(20
OH
(21)
OH
(22)
OH
(23)
OH
(24)

Figure 17. Structures of Compounds Having Trivial Names

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In gram-negative bacteria additional factors influence the antimicrobial properties of a compound: ad- and desorption of an active compound at cellular lipids (LPS, outer membrane) may occur in proportion to its lipophilicity. In fungi a great variation of cellular components, such as lipids, exists (*101,110*), which similarly cause loss of compound material.

The slopes of the calculated regression lines ranged from x = 0.499 to 0.755 with an average of 0.641 in gram-positive bacteria. The average slope was lower in gram-negative bacteria (x = 0.353 to 0.776, average 0.555,) and fungi (x = 0.212 to 0.647, average 0.413). This points to a stronger, lipophilicity dependent progression of toxicity in gram-positive bacteria than in gram-negative bacteria and fungi.

The lowest correlation coefficient was found in *Str. mutans*, which may find its explanation in the surface characteristics of this organism: biofilm formation, polysaccharide capsule, and synthesis of large amounts of extracellular mucoid glucans from sucrose (*100*). The comparatively low slope (x = 0.212) and correlation coefficient ($r^2 = 0.570$) in *A. niger* may point to an adaptation process towards phenols as consequence of the habitation in plant environment, e.g. *A. niger* utilizes phenols for pigment production (*107*). In contrast, the anthropophilic and susceptible fungus *T. mentagrophytes* ($r^2 = 0.787$, slope 0.647) exclusively infects humans and has no history in the utilization of phenols.

In the determination of the optimum lipophilicity of MOAPs towards each of the organisms examined the data situation was not always satisfactory due to partially missing MIC/logP - data pairs within the spectrum of logP from 1.5 to 10. In some cases inhibitory data of primary aliphatic alcohols confirmed or supplemented the data material of MOAPs. For this reason lipophilicity optima of *S. epidermidis, K. pneumoniae, P. acnes, L. monocytogenes and Str. mutans* are only tentatively characterized (Table **XVII**).

This unsatisfactory data situation influenced the decision to use linear and not binominal regression analysis in the statistical data examination. Especially in the case of *C. albicans* many data points were available close to $\log P_0$ and as a result a flattening of the curve is obvious. Recalculating these data with binominal regression analysis increased the correlation coefficient markedly from 0.562 to 0.746 ($\log 1/MIC = -0.750 + 1.989 * \log P - 0{,}238 * \log P^2$, n = 35, $r^2 = 0.746$). To obtain more precise information on the appearance of standard lines or curves a microbiologically recording of activities close to $\log P_0$ under constant testing conditions would be generally desirable.

Further compounds show antimicrobial activity in a logP range that is normally untypical for gram-negative bacteria as it can be taken from the Figures (**1**) to (**16**): 4-alkyl-2-benzylphenols (butyl, logP = 5.6; hexyl, logP = 6.6) in *E. coli* and *Ps. aeruginosa*. The same compounds cause strong growth inhibition in gram-positive bacteria (butyl: *M. luteus, S. aureus*, hexyl: *E. faecalis, S. aureus*). A similar observation is done with 6-alkyl-naphthalene-2-ol derivatives (logP =

4.2 to 5.3), which inhibited gram-positive (*S. aureus, S. epidermidis)* and surprisingly the gram-negative bacterium *Sal. typhi.* Another compound, 2-isopropyl-4-(6-isopropyl-4-methyl-1-cyclohexenyl)-5-methylphenol (**17**, logP = 7.5), contains a 2-isopropylphenol substructure, which was found to be important in the peptidoglycan synthesis inhibition by pisferic acid (**1**). Like totarol (**11**) a series of compounds (**2-8**) related to pisferic acid (**1**) presumably act as peptidoglycan synthesis inhibitors. A few ortho-tert.-butylphenols showed promising activity against *Str. mutans*. A selective mechanism of action seems to be probable for all of these compounds mentioned above.

By means of interpretation of regression lines and definition of optimum lipophilicities one should be able to distinguish between lipophilicity based, unspecific and specific, mechanism-based mode of antimicrobial action.

If the results from regression analysis of MOAPs can be used to identify mechanism-based antimicrobials - by considering laboratory MIC and the chemical structure of any compound - it should be possible to identify anti-infective drugs with known mechanism of action. To prove this hypothesis, the inhibitory and logP data of imidazole antifungal drugs were compared to the regression line and $\log P_0$ calculated for a fungus. *C. albicans* was chosen as model microorganism in this examination (Table **XVIII**). Because imidazole antifungals also inhibit gram-positive bacteria, the respective data of a microorganism of this type (*S. aureus*, Table **XIX**) were scheduled together from literature and the results on both tests are compared to each other (Figure **18**).

Thus, the bacterium *S. aureus* behaves towards imidazole antifungals like it does towards MOAPs: slope and intercept of eq 7 are very close to eq 18 as it can be seen from the regression lines additionally. With the fungus *C. albicans* the $\log P_0$ is 5 and further, unspecific acting compounds of higher lipophilicity were weak or not inhibitory. The activity of imidazole antifungals in the higher lipophilicity range of logP >5 (Eq 18) cannot be expected from eq 16 and $\log P_0$ definition. The dependence of antifungal activity from logP of azole antifungals is described best parabolically (Figure **18**).

But how to explain the diversity of these findings ? Firstly, an unspecific membrane action is unlikely and secondly, it appears to be a normal fact that these compounds were adsorbed by cytoplasma membrane due to their lipophilicity. In membrane permeation studies using lipophilic compounds on MDCK cell lines it was found that desorption from the lipophilic biomembrane is the key step in entering the cytosol. Desorption continuously decreased for molecules having logP of 4 to 7.7 (*142*). At a logP of approx. 8 desorption may not occur as it can be concluded by delineation of the correlation between transmonolayer permeability and lipophilicity (calculated CLogP) of pyrrolopyrimidines. This is supported by the calculation of lipophilicities of phospholipids, as they occur in cytoplasmic membranes, which is about 10 (e.g. di-palmitoyl-3-sn-phosphatidylethanolamine: XlogP = 10.499, (*26*)).

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Table XVIII. MIC and logP Data of Azole Antifungal Drugs Inhibiting *C. albicans*

Azoles (o)	*MIC*	*logP*	*lgMIC*	*Ref*
Oxiconazole	11.00	6.80	4.59	(*127*)
Fenticonazole	8.00	6.70	4.76	(*128*)
Sulconazole	1.00	6.23	5.60	(*128*)
Omoconazole	1.90	6.06	5.35	(*129*)
Miconazole	6.25	5.80	4.82	(*130*)
Clotrimazole	6.25	5.25	4.74	(*130*)
Econazole	16.00	5.09	4.38	(*128*)
Bifonazole	3.00	5.00	5.01	(*131*)
Chlormidazole	10.00	4.33	4.41	(*132*)
Enilconazole	100.00	3.74	3.47	(*133*)

Table XIX. MIC and logP Data of Azole Antifungals Inhibiting *S. aureus*

Azoles (o)	*MIC*	*logP*	*lgMIC*	*Ref*
Oxiconazole	3.10	6.80	5.14	(*134*)
Fenticonazole	2.00	6.70	5.36	(*135*)
Sulconazole	1.56	6.23	5.41	(*136*)
Omoconazole	10.00	6.06	4.63	(*137*)
Miconazole	1.50	5.80	5.44	(*138*)
Clotrimazole	3.00	5.25	5.06	(*139*)
Econazole	1.56	5.09	5.39	(*136*)
Bifonazole	25.60	5.00	4.08	(*140*)
Chlormidazole	40.00	4.33	3.81	(*142*)
Enilconazole	100.00	3.74	3.47	(*133*)

One can conclude that ergosterol (logP = 9), which presents an essential, unbond constituent of the phospholipid bilayer in fungal cells, cannot diffuse outside the membrane due to its lipophilicity. The same should be true for other compounds having similar physical properties. A few highly lipophilic antimicrobials are described in literature, e.g. clofoctol (2-(2,4-dichlorobenzyl)-4-(1,1,3,3-tetramethylbutyl)phenol, logP = 8.5) specifically inhibits energy metabolism in the cell membrane of *B. subtilis* (*143*), while anacardol derivatives (logP = 8 to 10) may disturb structure and essential activities within the cell wall of gram-positive bacteria (*51,52,144*).

$$\text{Eq: } \log 1/\text{MIC} = -6.652 + 3.961 * \log P - 0.334 * \log P^2, \quad n = 10, r^2 = 0.723, s = 0.348 \qquad (17)$$

$$\text{Eq: } \log 1/\text{MIC} = 1{,}601 + 0.578 * \log P, n = 10, r^2 = 0.612, s = 0.488 \qquad (18)$$

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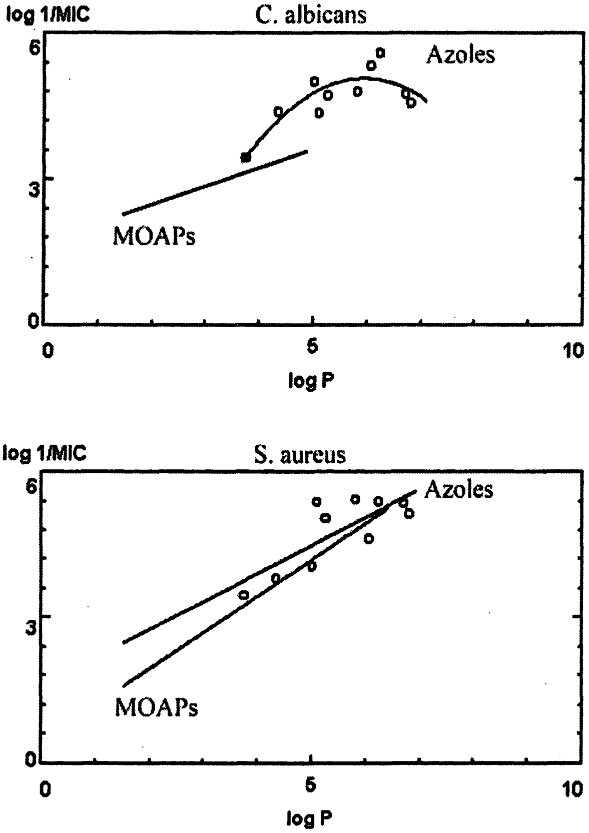

Figure 18. Dependence between MIC and logP of MOAPs and Imidazoles inhibiting C. albicans (Eq 16,17) and S. aureus (Eq 7,18).

The strong inhibitory azoles listed in Table **XVIII** do not exceed a logP of 7. If the results obtained with membrane monolayers can be transferred directly to cytoplasmic membrane in fungal or eukaryotic cells in general, the high activity of imidazole antifungals can be explained with their ability to desorb from the cytoplasmic membrane, their diffusion through the cytosol to the endoplasmatic reticulum, renewed absorption to this second, intracellular biomembrane (passive transport, 5.000 dalton maximum molecule size (*145*)) and finally, selective inhibition of lanosterol-14a-demethylase mediated removal of the C14-methyl group in lanosterol, the target mechanism of azole antifungals (*146,147*). Passive membrane transport and increase of inhibitory activity with increase of solubility in fat has been already described with azole antifungals (*148*). A specific mechanism of action can be proposed for azole antifungals when using eq 16 and $\log P_0$ definition in the analysis of inhibitors of *C. albicans*.

Similarly compounds having a specific mechanism of action can be selected by using eqs 1 to 16, which describe the antimicrobial activity of MOAPs towards various kinds of microbial species. The regression lines are useful as standard values for a given microbial species, respectively. A systematic recording of such standard values for a greater number of microorganisms under constant experimental conditions is desirable. Nevertheless, the chemical information given by the compound structures is another important source in development of new leads (*149*). Both, the information on physical properties and chemical structure of antimicrobials should be brought together in a computer database that carries out mathematical operation to analyze the data material.

Finally, three types of lipophilic antimicrobials can be characterized by the aforementioned physical properties of cellular components and partitioning within the microbial cell:

I) unspecific acting lipophilic compounds that solute in the cytoplasmic membrane and cause destruction of the membrane itself or its vital components ($\leq \log P_0$). The optimum lipophilicity for such compounds is characterized with $\log P_0 = 4 \pm 0.5$ in gram-negative bacteria, $\log P_0 = 5$ in fungi and $\log P_0 = 6 \pm 0.5$ in gram-positive bacteria (details in Table **XVII**). Examples are thymol and carvacrol ($\log P = 3.2$), two naturally occurring phenols having a broad-spectrum of antimicrobial activity.

II) specific acting compounds having a logP of ≤ 7.5 to approx. 8 in maximum. Such compounds have the ability to desorb from the cytoplasmic membrane and to enter the cytosol in amounts that cause a decline of the microorganism. Examples are the well studied azole antifungals ($\log P_{\text{max.}}$ 6.8, $\log P_0$ *C. albicans* = 5), pisferic acid and related compounds (**1-8**, logP range 6.7 to 7.7, $\log P_0$ gram-positive bacteria ~ 6).

III) specific acting compounds having a $\log P > 8$. Such compounds may not desorb from the cytoplasmic membrane and may not enter the cytosol in amounts that cause a decline of the microorganism. Type III compounds specifically impair vital functions within cytoplasmic membrane or the cell wall. Examples are anacardol derivatives (logP range 8 to 10) and the drug clofoctol ($\log P = 8.5$) (*143,150*).

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Chapter 12

Where Old Biocides Meet New Biocides: Hypohalite Defense Factors of the Human Oral Cavity

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The foci of this report are two endogenous hypohalites that have been proposed to control the oral cavity, hypochlorite (OCl^-) and hypothiocyanite ($OSCN^-$). Hypochlorite is an archetypal biocide, whereas the chemistry and biological significance of hypothiocyanite are less well understood. Within the oral cavity, the hypohalites are generated by enzymes. However, we have observed a facile non-catalytic pathway that produces hypothiocyanite by oxidation of thiocyanate (SCN^-) with hypochlorous acid (HOCl), a reaction that preserves the oxidizing equivalents of hypochlorite by transferring them to hypothiocyanite, a more discriminate biocide that is not lethal to mammalian cells. Remarkably little is known about the biocidal mechanisms of the hypohalites, due in part to their extraordinary reactivities and the fact that they produce cascades of derivative intermediates with largely unknown physiologic properties. In addition to the complexities of the dynamic chemistry, the issue of biocidal mechanisms is further complicated by the heterogeneity of the oral cavity, wherein multifarious biofilms develop in chemically distinct environments. Given that these hypohalites apparently control different spatial environments of the oral cavity, but interfacial regions near the gingival margin (a battleground for biofilm formation) likely exhibit a concentration gradient of these hypohalites, we hypothesize that these two biocides may act in concert. We review herein

In New Biocides Development; Zhu, P.;
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the literature that pertains to the formation and distribution of hypohalites in the oral cavity, the chemistry that describes their subsequent reactions, and we discuss possible biological targets.

Introduction

There is resurgent interest in microbicides due to the emergence of antibiotic-resistant microorganisms. Particularly alarming are multi-drug resistant human pathogens. In contrast to antibiotics, microbicides do not exhibit a propensity for inducing resistance, because they target multiple intracellular pathways. Our research is focused on human non-immunological defense factors that strike a balance between microbicidal efficacy and host tissue damage. In particular, we are investigating a cascade of Reactive Sulfur Species (RSS) that begins with the two-electron oxidation of thiocyanate (SCN^-) to give hypothiocyanite ($OSCN^-$) (1, 2). In contrast to less discriminate biocides, the $OSCN^-$ cascade can be cytocidal toward prokaryotes, but it is apparently only cytostatic toward eukaryotes (3-7). Notably, $OSCN^-$ is a transient species that produces a cascade of RSS, and it is not known which of these species are actually biologically active. The primary mechanism of biosynthesis of $OSCN^-$ is enzymic oxidation of SCN^- by peroxidases (7-9). However, we have observed that powerful chemical oxidants, such as phagocyte-derived hypochlorous acid (HOCl) and hypobromous acid (HOBr), are capable of oxidizing SCN^- to $OSCN^-$ in near diffusion-controlled uncatalyzed reactions that may serve to protect host tissues by limiting the lifetimes of the less discriminate biocides (1, 2). While it is possible that the aforementioned reactions play important systemic roles in human health (1, 2), we focus our attention here on the oral cavity where the hypochlorite and hypothiocyanite defense mechanisms are generally spatially segregated, although the chemistries commingle at the gingival margin (gumline). Following a discussion of the role of hypohalites in human host defense, we will provide a progress report of our studies. Particular focus will be placed on describing the chemical mechanisms that interconvert halide (Cl^- and Br^-) and pseudo-halide (SCN^-) derived biocides. We also discuss the reaction chemistry of these biocides in the context of biological targets. We note that this is not a comprehensive review, and we apologize in advance to the researchers in the field whose work has been omitted.

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Health Consequences of Microbial Growth in the Oral Cavity

Approximately $6 billion is spent annually to treat periodontal disease, which afflicts 20 to 30 percent of all adults in the industrialized world. This is one of the most common chronic infectious diseases afflicting adults, and roughly 70 million adults are believed to be affected in the U.S. alone. Chronic inflammation and infection of the gums and surrounding tissue is the major cause of about 70 percent of adult tooth loss, affecting three out of four persons at some point in their lives. The oral bacteria which cause periodontal disease have also been linked to a number of systemic diseases including infective endocarditis (10), stroke (11), atherosclerosis (12, 13), myocardial infarction (14), and preterm low-birth-weight babies (15). The oral cavity, arguably the most complicated environment in the human body, provides a challenge for host defense, and fertile ground for microbial colonization. Adhesion is a necessary ecological factor for oral bacteria to thrive (16), and the mouth provides many distinctive surfaces including mucosal surfaces (e.g., tissues) and the enamel surfaces of teeth. The exposed soft tissues of the oral cavity do not encourage long-term adhesion of microorganisms (as a consequence of desquamation, mastication, salivary flow, and oral hygiene). However, the surfaces of teeth, and especially those surfaces that are not readily accessible, afford the opportunity for enduring microbial populations. These areas include fissures (deep, narrow grooves in teeth), approximal surfaces (the space between teeth), and the space between the surface of the tooth and the free gingiva (gingival crevice). Due to differences in their physicochemical properties, these environments provide optimal growth conditions for different microorganisms. Long-term colonization of these surfaces leads to the formation of biofilms (dental plaque). The defensive mechanisms of the oral cavity and other governing factors keep bacterial growth in check, but once established, resident microflora reach homeostasis. The resulting biofilms are comprised of a consortia of microorganisms, which are commensal in healthy states. However, for reasons that are not entirely understood, ecological pressures can result in microbial shifts whereby acidogenic and cariogenic Gram-positive bacteria replace pre-existing acid-sensitive species. At the same time, fastidious obligate anaerobes, including Gram-negative proteolytic species that are associated with periodontal diseases, begin to populate the subgingival crevice. While the etiology of dental diseases is complicated, it is clear that the predominant bacterial species that are recovered from diseased individuals are different from those that are found in healthly individuals (17). We will discuss next the role that host defense mechanisms, and in particular the hypohalite-generating peroxidases, play in curbing microbial growth in the oral cavity.

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Mechanisms that Produce the Hypohalite Host Defense of the Oral Cavity

There are two defensive peroxidases of the oral cavity (18), salivary peroxidase (SPO, a.k.a. sialoperoxidase and sometimes incorrectly as lactoperoxidase) and myeloperoxidase (MPO). Ironically, the tissue damage that gives rise to periodontitis is partially attributed (19, 20) to one of the two peroxidase systems of the oral cavity that are charged with host defense. While both of these peroxidase systems, SPO and MPO, typically oxidize SCN^- to produce $OSCN^-$, a selective antimicrobial that targets prokaryotic organisms and is comparatively chemically benign (3), MPO is also capable in certain physiologic fluids of generating the powerful biocide OCl^- (the principal component of household bleach) that can give rise to necrosis of gum tissue. MPO is the only mammalian enzyme that is capable of producing OCl^-. This Jekyll and Hyde personality of MPO and the intimate chemical relationship between the highly reactive hypohalite defense factors that involve both host defense and inflammatory disease speak to the need for models that describe their physiological chemistries and biocidal mechanisms in the diverse and heterogeneous environment of the oral cavity.

SPO is a normal non-inducible component of saliva, whereas MPO is one of two oxidative defense mechanisms of neutrophilic polymorphonuclear leukocytes (PMNs). Leukocytes are not normal components of the saliva of healthy individuals, but rather are introduced to the oral cavity by gingival crevicular fluid (GCF) during inflammatory response. PMNs typically comprise 33-75% of all leukocytes in humans, and MPO accounts for about 5% of the total PMN protein (21). While PMNs are typically the most abundant leukocytes and are the first to respond to sites of inflammation, macrophages generally play a larger role in most chronic inflammations. However, it is noteworthy that PMNs dominate throughout all stages of periodontal disease (20). About 80% of the PMNs are viable and functional within the crevice. These cells are capable of phagocytosis and of killing microorganisms, although the efficiency of phagocytosis is reduced compared with that of blood PMNs (22). As a consequence of the polymeric matrix that envelops the bacteria of oral plaque (and other factors), the phagocytic mechanism of PMNs is relatively ineffective toward biofilms. PMNs may remain functional at a short distance from the gingival margin by flow of GCF along the tooth surface, but PMNs can degenerate in saliva due to osmotic lysis, thereby releasing the content of the auzurophilic granules (including MPO).

As mentioned above, SPO and MPO are capable of catalyzing the oxidation of halides (X^-) to hypohalites (XO^-) for the purpose of cellular defense. The mechanism of oxidation by these enzymes appears to be similar: reaction of the

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Table 1. Two-electron Redox Couples for X^- (E^{o}', pH=7 vs. SHE), Rate Constants (k_2) of MPO Compound I ($x10^4M^{-1}s^{-1}$) with X^-, Reference Range Values of X^- in Physiologic Fluids (μM or mM), and Specificities (S)[a] for Oxidation of X^- by MPO. Consensus Substrates in Bold.

X^-	E^o	k_2[b]	RRV in GCF[c]	GCF S	RRV in Saliva	Saliva S
Cl^-	1.08	2.5	**90 mM**	**6**	25 mM	1
SCN^-	0.77	960	40 μM	1	**1 mM**	**15**

[a] $S = k^{X^-_{maj}}[X^-_{maj}]/k^{X^-_{min}}[X^-_{min}]$. [b] Furtmüller, P. G.; Burner, U.; Obinger, C. *Biochem.* **1998,** *37,* 17923. [c] Anttonen, T.; Tenovuo, J. *Proc. Finnish Dental Soc.* **1981**, *77*, 318.

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ferriheme enzyme with H_2O_2 (23) to form Compound I (PO-I, which contains two oxidizing equivalents more than the resting enzyme), followed by direct reaction of the halide with Compound I in a two-electron process to produce hypohalite (Eqns 1-2):

$$PO + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PO\text{-}I \qquad (1)$$

$$PO\text{-}I + X^- \xrightarrow{k_2} PO + OX^- \qquad (2)$$

The hypohalites are much more effective antimicrobials than H_2O_2. Thiocyanate appears to be the only physiological substrate for SPO (and LPO). The preferred substrate for MPO depends upon two factors, the relative rates of reaction of Compound I with the substrates and the bioavailabilities of the substrates (Table 1). The principal substrates for MPO in physiologic fluids are Cl^- and SCN^-. Although MPO is capable of employing Br^- and I^- as substrates, the concentrations of these halides are too low for them to be competitive. Thiocyanate is roughly 1000 times more reactive than Cl^-, but the physiological concentration of Cl^- is typically 1000 times greater than SCN^-. Accordingly, OCl^- and $OSCN^-$ are produced by MPO in roughly the same magnitude of concentration, and any preference depends upon the relative concentrations of Cl^- and SCN^- in various physiologic fluids. Table 1 summarizes the substrate selectivities of MPO in GCF (similar in composition to plasma) and in saliva. Chloride is a competitive substrate for MPO when the concentration of SCN^- is relatively low (e.g., in GCF, Table 1) (24). In contrast, SCN^- is the preferred substrate in saliva (Table 1).

We have recently shown (1) that HOCl oxidizes SCN^- to give $OSCN^-$ in a reaction that is nearly diffusion-controlled under physiological conditions (Eqns 3-4, X = Cl, $k_4 = 2\times10^7\ M^{-1}s^{-1}$):

$$OX^- + H^+ \xrightleftharpoons[k_a]{k_a} HOX \tag{3}$$

$$HOX + SCN^- \xrightarrow{k_4} X^- + OSCN^- + H^+ \tag{4}$$

Accordingly, the substrate selectivity of MPO may not be an issue if target reductants do not react with HOCl with rates that are kinetically competent with respect to its reaction with endogenous SCN^-, *vide infra* (1).

The concentration of SCN^- in saliva is higher than in any other extracelluar fluid, due to active transport mechanisms (25). Thus, not only will MPO produce less OCl^- in saliva versus GCF (Table 1), but the lifetime of the OCl^- that is produced will be less in saliva versus GCF because of its reaction with SCN^- (Eqns. 3-4). Using the concentrations of SCN^- in Table 1, we compute

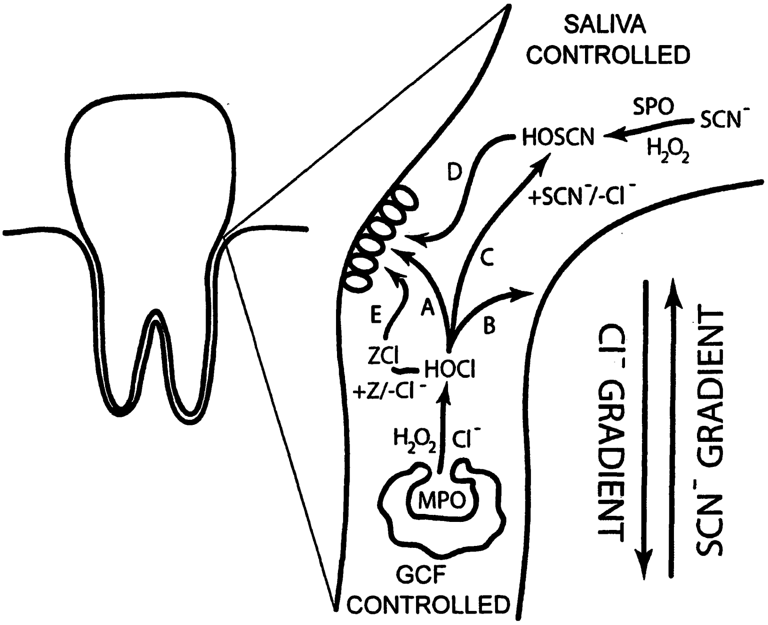

Figure 1. Spatial relationship between the inorganic host defense factors of the oral cavity and the ion gradients that influence their relative abundance.

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half-lives for OCl^- in saliva and GCF of ~35 μs and ~1 ms, respectively. This conclusion is reinforced by the observation by Tenovuo, *et al.* that SCN^- attenuates the antibacterial effectiveness of MPO (26). In effect, the only antimicrobial product of the SPO and MPO systems in saliva is expected to be $OSCN^-$ (and possibly its derivatives), but a continuum of products are expected at the gingival margin where a gradient of concentration of Cl^- and SCN^- exists.

The spatial relationship between these gradients and the areas of the oral cavity that are respectively controlled by the OCl^- and $OSCN^-$ defense factors are illustrated in Figure 1 (previous page). In summary, extraphagosomal HOCl is cytotoxic to pathogenic bacteria (Path A) and gingival tissue (Path B). Alternatively, HOCl can react with SCN^- to produce HOSCN (Path C). HOSCN is also produced during the SPO-catalyzed oxidation of SCN^- by H_2O_2. HOSCN is antibacterial (Path D, but comparatively innocuous with respect to the host tissues). In addition to the reaction of HOCl with SCN^-, it may react with other small molecules (Path E) to produce secondary antimicrobials (e.g., organic amines to produce chloramines that are generally cytotoxic). Path E is perhaps the least understood bacteriocidal mechanism of HOCl.

Transitory Nature of the Hypohalites

Hypochlorite

The chemical properties of hypochlorite are well understood. It is thermodynamically unstable in water and will eventually undergo inorganic disproportionation reactions to yield a stoichiometric mixture of Cl^- and perchlorate (ClO_4^-) vis-à-vis a cascade of intermediate oxoanions (27). However, HOCl is a powerful electrophilic chlorinating agent, and in the mélange of reactive nucleophiles that exist in physiologic fluids, the kinetics of the formation of organic chlorine derivatives of HOCl are certain to be more competitive than the aforementioned inorganic reactions (28-30). The reactions of HOCl with thiols, thioethers, and amines are particularly facile. With respect to biocidal mechanisms, the reactions of HOCl with amines are particularly relevant because chloramine derivatives are generally cytotoxic and because some derivatives may play a role during inflammation of the oral cavity (19).

Hypothiocyanite

Despite the implication of $OSCN^-$ as a major player in host defense of the oral cavity (and elsewhere), remarkably little detail is available about the

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chemistry of this species, and even less is known about the mechanism of antimicrobial activity. Hypothiocyanite is actually a complicated equilibrium mixture of several inorganic species in aqueous solution (1, 31-34). Hereafter, we refer to this mixture as "hypothiocyanite" (in quotes). Until recently, there was relatively little quantitative information about the equilibria of "hypothiocyanite". The pK_a of HOSCN is believed to be 5.3 (Eqn 5) (35, 36). Stanbury, *et al.* have published several landmark papers that have provided insight into the nature of "hypothiocyanite" in acidic (e.g., 1 M H^+) aqueous solution (32-34). A key breakthrough in Stanbury's work was the observation that aqueous solutions of Cl_2 rapidly and cleanly oxidize SCN^- to give equilibrium mixtures of "hypothiocyanite". Given what we know about the effects of pH on the equilibrium $Cl_2 + H_2O = HOCl + HCl$, it is not surprising that the analogous hydrolysis of $(SCN)_2$ to give HOSCN has a small equilibrium ratio under acidic conditions (Eqn 6):

$$\text{HOSCN} \rightleftharpoons \text{OSCN}^- + \text{H}^+ \qquad (5)$$

$$(\text{SCN})_2 + \text{H}_2\text{O} \rightleftharpoons \text{SCN}^- + \text{HOSCN} + \text{H}^+ \qquad (6)$$

$$(\text{SCN})_2 + \text{SCN}^- \rightleftharpoons (\text{SCN})_3^- \qquad (7)$$

Thus, the principal species in "hypothiocyanite" mixtures under acidic conditions is $(SCN)_2$ ($K_6 = 6\text{x}10^4\ M^2$) Furthermore, Stanbury has presented convincing evidence that $(SCN)_3^-$ is formed in the presence of excess SCN^- (Eqn 7, $K_7 = 0.4\ M^{-1}$, cf. I_3^-). Accordingly, the electronic spectrum of acidic aqueous solutions of "hypothiocyanite" is dominated by the spectrum of $(SCN)_3^-$ in the presence of even a small excess of SCN^- ($\varepsilon((SCN)_2)_{300nm} = 75\ M^{-1}cm^{-1}$ and $\varepsilon((SCN)_3^-)_{300nm} = 9500\ M^{-1}cm^{-1}$). Stanbury's most recent paper on this subject describes the kinetics of decomposition of $(SCN)_2$ under acidic conditions (31). Since $(SCN)_2$ and $(SCN)_3^-$ probably do not exist in significant amounts at physiologic pH, we have recently begun to investigate the chemistry of "hypothiocyanite" under more basic conditions, which are conditions in which the species $OSCN^-$ should dominate. The results of these studies have either appeared in print (1, 2, 37, 38) or the salient results are described next. We have measured the rates of reaction of HOX (X = Cl and Br) with SCN^- (1, 2). HOBr, the putative killing agent of eosinophil peroxidase (39-42), reacts nearly two orders of magnitude faster with SCN^- than HOCl does ($k = 2\text{x}10^9\ M^{-1}s^{-1}$) (2), at the diffusion-controlled limit. We have proposed that the scavenging of HOX by SCN^- may play an important physiological role in redox buffering the powerful hypohalite biocides (1, 2). We have also sought to exploit the facile reaction of HOX with SCN^- to investigate the kinetics and mechanisms of the inorganic chemistry of "hypothiocyanite". Our publications thus far have focused on alkaline pH. Such conditions slow the reactions of HOX with SCN^- sufficiently to permit their investigation under conditions of stopped-flow mixing. Since we have already published the kinetics of the reactions of Eqns 3 and 4 (1, 2), we concentrate here on our efforts to characterize the product, which we have presumed to be $OSCN^-$.

All of the experiments we report here have been carried out at pH 13 (0.1 M NaOH) unless otherwise noted. We focus first on our evidence that $OSCN^-$ is the initial product of the reaction of HOX and SCN^-:

- The electronic spectrum of the initial product (Figure 2) is the same regardless of whether HOCl or HOBr is employed as the oxidant.
- After the reaction of $OSCN^-$ at pH 13, acidification to pH 0.3 produces the (quantitative) electronic spectrum of $(SCN)_2/(SCN)_3^-$ (cf. the work of Stanbury, *et al.*).
- The kinetics upon acidification are first-order in $[SCN^-]$ and $[H^+]$ and independent of HOX, as expected from Eqn 6.
- The same initial ^{13}C NMR spectrum (δ = 127.8 ppm relative to dioxane at 66.6 ppm) is obtained for the reaction of SCN^- with HOX and the hydrolysis of $(SCN)_2$ (a reaction that is expected to yield $OSCN^-$ vis-à-vis Eqn 6).
- The kinetics of decomposition of $OSCN^-$ and the products are the same (*vide infra*) regardless of whether it is synthesized by the HOX or the $(SCN)_2$ route.
- $OSCN^-$ reacts with cysteine with second-order kinetics and a 1:1 stoichiometry.

We note that the ^{13}C spectrum we have obtained is comparable to one that has been previously reported for the LPO-catalyzed oxidation of $SCN^-$by H_2O_2 (43). Also, the electronic spectrum we have obtained is analogous to literature spectra $OSCN^-$ that has been prepared by the enzyme route. However, the non-enzymic route we have employed and the alkaline conditions have permitted us to obtain more concentrated solutions of $OSCN^-$ than that have been previously possible using enzymic methods, and this has permitted us to identify new spectral features in the electronic spectrum (Figure 2). In summary, the product of the reaction of HOX with SCN^- at pH 13 has the spectral and chemical properties we expect for $OSCN^-$.

In the absence of excess HOX or other reactive species (e.g., thiols), HOSCN apparently decomposes to yield a cascade of transitory derivatives. While the precise chemical natures of these derivatives are presently under investigation, we have some information regarding their lifetimes and some of the factors that influence the rates of their decomposition. It is apparent from our preliminary investigations that the mechanisms of the decomposition pathways of $OSCN^-$ are very complicated at neutral pH. One reason for this may be the fact that many of the reactions are close to being diffusion controlled. Such

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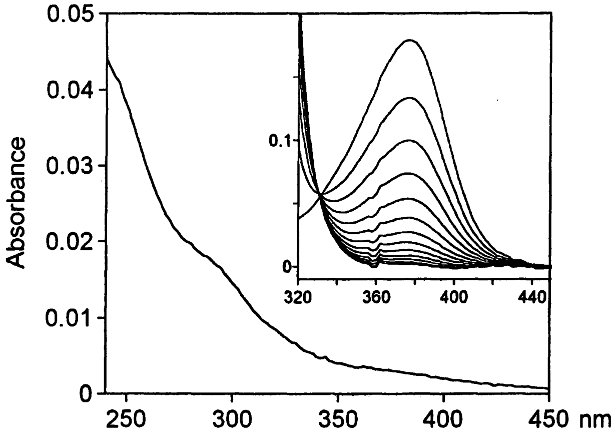

Figure 2. Electronic spectrum of $OSCN^-$ *(40 μM) in 0.1 M NaOH as generated from the reaction of* OCl^- *(40 μM) with* SCN^- *(400 μM). The spectrum of excess* SCN^- *(360 μM) has been subtracted. Inset: Time-resolved spectra of the decomposition of* $OSCN^-$ *(*$[OSCN^-]_0$*=10 mM), with 200 mM* SCN^- *background subtracted, illustrating the disappearance of the band at* λ_{max}*=376 nm.*

reactions occur before complete mixing of reactions can take place in a stopped-flow experiment, which can give rise to high local concentrations that facilitate reaction pathways (e.g., over oxidation) that would not occur otherwise. We will once more focus here on our observations at pH 13, where the decomposition of $OSCN^-$ appears to be a clean, albeit unexpected reaction. We have measured the rate of decomposition of $OSCN^-$ by UV-visible spectroscopy and ^{13}C NMR spectroscopy. The rate of decomposition, which is a relatively slow process ($k = 4 \times 10^{-4}\ s^{-1}$; $t_{1/2} = 29$ min), is first order in $[OSCN^-]$ and independent on the $[SCN^-]$. This observation is not consistent with disproportionation or condensation reactions, which we would expect to be second-order in $[OSCN^-]$. Our investigation of the product of the decomposition of $OSCN^-$ at pH 13 is not yet complete, but it is clear that its spectral and chemical properties are different that the products that are observed at lower pH. It remains to be shown whether the decomposition reaction we observe for $OSCN^-$ at pH 13 is relevant to the physiological chemistry of $OSCN^-$.

Interconversion of the Halogens, Hypohalites, and Interhalogen Compounds

While the discussion thus far has largely focused on the production of the hypohalites via primary processes (i.e., enzyme catalyzed oxidation of halides),

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it is very likely that under some circumstances the resulting hypohalites will undergo subsequent inorganic reaction to produce secondary reactive species that in some cases may be more effective biocides that the original hypohalites. For example, the interhalogen compound bromine chloride (BrCl) is formed in equilibrium mixtures when HOCl reacts with Br^- (44), and there is mounting evidence that this species may figure significantly in the biological chemistry of HOCl (45, 46). While the substrate selectivity of MPO is such that Cl^- and SCN^- are the preferred substrates under physiologically relevant halide concentration (Table 1, cf. eosinophil peroxidase (EPO)), subsequent reaction of HOCl with Br^- can yield BrCl. It is noteworthy that the relative equilibrium concentration of BrCl goes up markedly at lower pH (Figure 3) and that mature oral biofilms frequently exhibit a local pH less than 4.5 (47). Figure 3 illustrates the speciation of halogens and oxyacids that are produced from a solution that contains 100 mM Cl^- and 50 μM Br^- (typical concentrations of these ions in human blood plasma) when 50 μM HOCl is added. Note that the principle species in solution is HOBr, and HOBr is typically two orders of magnitude more reactive than HOCl, *vide infra*. We note also that BrCl is typically an even more reactive electrophilic brominating agent than HOBr. The influence of Br^- on solutions of OCl^- has been taken advantage of by commercial manufacturers of disinfectants. For example, the popular disinfectant Stabrex (Nalco) is a formulation of 11% NaOCl and 15% NaBr. Importantly, hypohalite disinfectant formulations, including bleach, are delivered at alkaline pH since they are

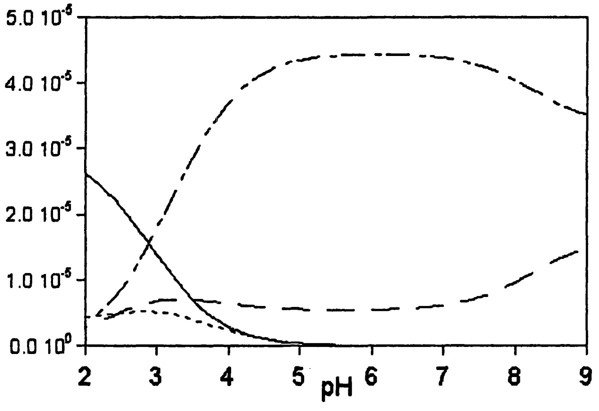

Figure 3. Speciation of halogens and oxyacids at a function of pH for $[Cl^-]$ *= 100 mM,* $[Br^-]$ *= 50 μM, and* $[HOCl]_0$ *= 50μM: Solid = BrCl, Dot-Dashed =* $HOBr+OBr^-$*, Long Dashed =* $HOCl+OCl^-$*, and Short Dashed =* Br_2*. The minor species* Cl_2*,* Br_2Cl^-*,* $BrCl_2^-$*, and* Cl_3^- *are omitted from the figure (but not the calculation) for the sake of clarity.*

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unstable near neutral pH. Thus, the formulations themselves do not contain halogens (and Stabrex does not contain BrCl), but the lowering of pH upon use can generate such species.

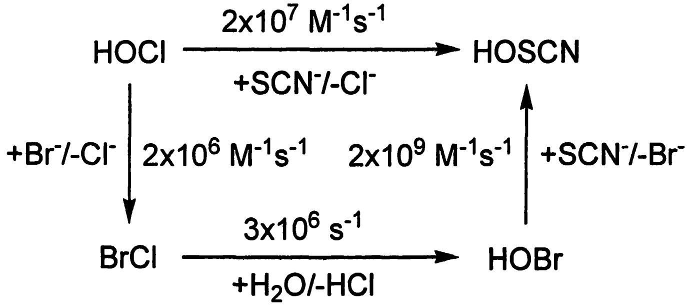

Figure 4. Alternative reaction pathways for the formation of hypothiocyanite.

It is important to point out that Figure 3 illustrates the thermodynamic equilibrium distribution of Cl and Br-containing species. However, some of the reactions that interconvert these species may not be kinetically competent with respect to the reactions of the precursors with other species in the reaction medium. Figure 4 summarizes the rate constants for a few relevant reactions. While Figure 3 suggests that HOBr/OBr^- would be the predominant species at physiologic pH, and the two-order-of-magnitude larger rate constant for the reaction of HOBr with SCN^- versus the corresponding reaction with HOCl would be favorable, Figure 4 illustrates that the interconversion of HOCl to HOBr via the intermediate BrCl is rate-limiting, and thus HOCl that is generated in a plasma-like environment would be expected to react with SCN^-. A similar conclusion is reached for alternative pathways for the interconversion of HOCl and HOBr (e.g., the direct reaction of HOCl with Br^- to give HOBr, which has a rate constant of $2x10^3$ $M^{-1}s^{-1}$). Furthermore, the scavenging of HOCl by SCN^- would be even more efficient in a saliva-like environment that contains higher concentrations of SCN^-. Thus, we do not anticipate that Br-containing species play an important role in the oral cavity.

Reactivity Patterns of the Hypohalites

In general, the most effective scavengers of HOCl and HOBr are organosulfur compounds, and in particular, cysteine (Cys) (28, 29, 48) and methionine (Met) (49) derivatives, including the small tripeptide glutathione (GSH), which exhibits millimolar cytosolic concentrations in human cells (e.g., 2 mM in erythrocytes) (50) and macromolecules, such as human serum albumin

(HSA), which bears a single reduced Cys group on its surface, six Met groups, and is abundant in plasma (ca. 300 μM) (51). HOBr, and to a lesser extent HOCl, also exhibit significant reactivity toward N-H moieties and aromatic residues. Pattison and Davis have reported the second-order rate constants for the reaction of HOX with proteinaceous components. We summarize those rate constants together with those we have measured for SCN^- in Figure 5. Figure 6 illustrates a model for the partitioning of HOX (X = Cl and Br) between the free amino acids in human blood plasma, as previously proposed by Pattison and Davies (52) (unfilled bars). This model predicts that HOCl will react principally with sulfur-containing components, whereas HOBr will react largely with nitrogen-containing species. We have augmented this model by including the effects of SCN^- using a normal reference value of 50 μM (hashed bars). The effect of SCN^- on the partitioning of HOCl is minimal, but a marked influence on the partition of HOBr is predicted because SCN^- is expected to compete

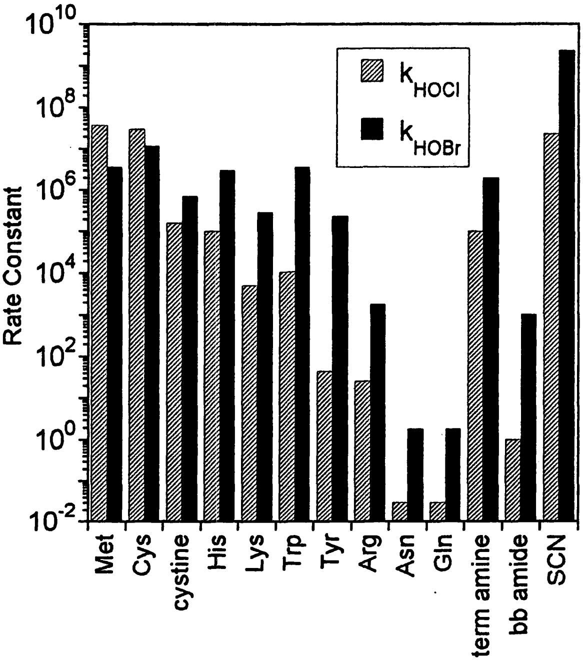

Figure 5. Summary of the effective second-order rate constants (log scale and corrected for pH 7.4) for the reactions of HOCl (hashed bars) and HOBr (filled bars) with various proteinaceous components and thiocyanate. Adapted from Pattison, D. I.; Davies, M. J., Biochem. ***2004****, 43, 4799. Note the general trend of ca. 100-fold larger rate constants for HOBr as compared with HOCl, with the notable exceptions of Cys and Met.*

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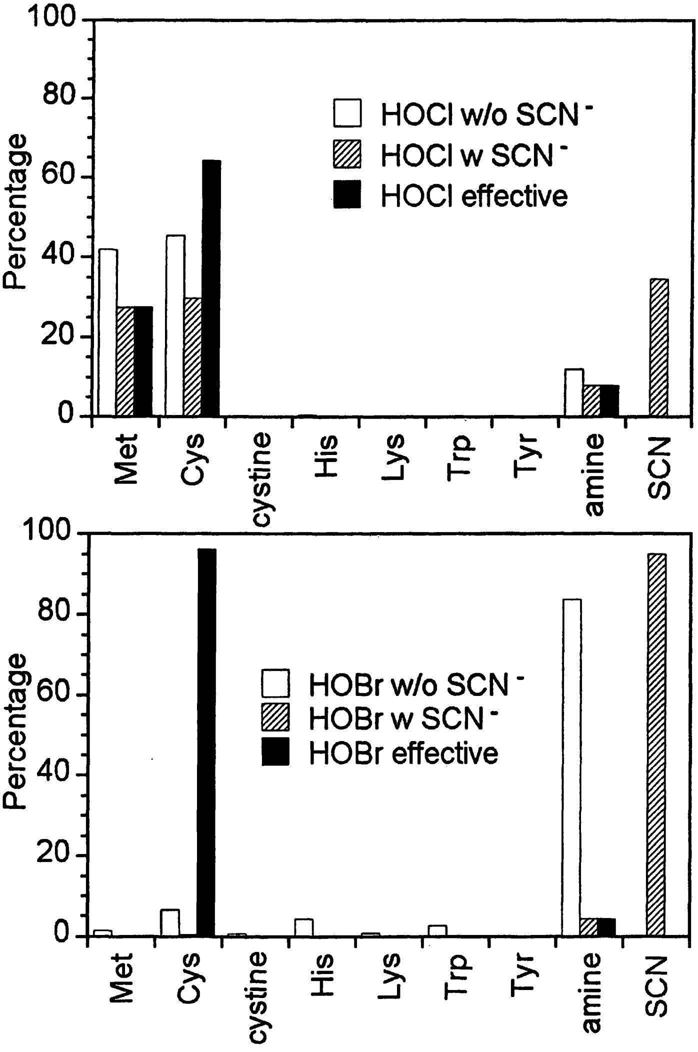

*Figure 6. Histogram illustrating the expected partitioning (initial rates) of HOCl (top) and HOBr (bottom) between free amino acids and thiocyanate in plasma using three models: 1. the original model as proposed by Pattison and Davies (Biochem. **2004**, 43, 479) (unfilled bars), 2. the same model with the inclusion of SCN^- with a normal reference value of 50 μM (hashed bars), and 3. the latter model after taking into account that the $OSCN^-$ that is produced by the reaction of HOX and SCN^- is expected to subsequently react with Cys to yield, after hydrolysis, effective oxidation (filled bars).*

effectively for most of the HOBr, presumably to produce $OSCN^-$. Given the apparent selectivity of $OSCN^-$ for thiols (37, 53-55) in a reaction that effectively yields the same oxidation products that would have resulted if HOBr had reacted directly with Cys, the anticipated net effect of the intervening SCN^- is to cause the oxidation of Cys (solid bars), not nitrogen-derivatives as predicted in the original model. Importantly, we do not intend Figure 6 to be a model for the partitioning of HOX in a plasma-like environment. On the contrary, this example illustrates the challenges faced in developing models of physiological chemistry, wherein the addition of a single parameter significantly alters the outcome. We are of the opinion that mechanisms are never proven, but rather alternative mechanisms can be ruled out (56, 57). Thus, mechanisms should be continuously refined as new information becomes available (57). And in this context, we note that SCN^- is not the only species in GCF that is expected to scavenge OX^-. Recent investigations have suggested that GCF contains GSH concentrations that are 1000-fold higher (0.5-2.5 mM) than those normally found in extracellular fluids (58). However, it is noteworthy that the GCF of individuals with periodontitis exhibits concentrations of GSH that are lower than that found in the GCF of healthy individuals.

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Biocidal Mechanisms and Synergism of the Hypohalites

Hypochlorite has been studied as a killing agent both from the perspective of a disinfectant (59) and of a human defense factor (60). The mechanism of action of hypochlorite has been explored for vegetative prokaryotes (61) and their spores (62) as well as eukaryotes (63). Given its promiscuous reaction chemistry (Figure 5), it is not surprising that hypochlorite affects many cellular functions of bacteria. In general, the cellular response to insults by hypochlorite (64-66) are similar to those of other oxidizing agents (67). In contrast to the plethora of studies that have involved hypochlorite, there have been comparatively few investigations of the mode of action of hypothiocyanite as a biocide. We summarize here a few general observations regarding hypothiocyanite and its role as a biocide:

- "Hypothiocyanite" reacts (exclusively) with sulfhydryl groups (37, 53-55).
- "Hypothiocyanite" is inhibitory, but not cytotoxic, to eukaryotic cells (3-7).
- Not all prokaryotes are affected by "hypothiocyanite" (3-7).
- The cytotoxic properties of "hypothiocyanite" are dependent on pH (68-71).
- The availability of H_2O_2 (not enzyme or SCN^-) limits the concentration of "hypothiocyanite" *in vivo* (72-75).
- Peroxidases that produce "hypothiocyanite" may protect hosts from H_2O_2 cytotoxicity (1, 3-7).

Regarding the first point, we note that about 40% of all enzymes are abolished by reagents that bind to SH groups (76), thus providing a basic hypothesis for the cytotoxicity of "hypothiocyanite". Furthermore, recent studies on the stress response of *Escherichia coli* to the lactoperoxidase-thiocyanate system using reporter gene assays have identified promoters that are specifically induced (77-81). These studies suggest that "hypothiocyanite" solicits a stress response that is quite different from that which is induced by hypochlorite. These observations have lead us to believe that the hypochlorite and hypothiocyanite host defense mechanisms may act synergistically. In retrospect, it is perhaps not surprising to learn that hypochlorite and hypothiocyanite operate by different mechanisms given their dissimilar reaction properties.

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A Continuum of Biocidal Activity in Combating Biofilms

We theorize that the actual antimicrobial components of the hypothiocyanite system may include secondary RSS that are produced upon decomposition of $OSCN^-$, and that such species may be more effective antimicrobials for human bacterial biofilms (Figure 7). One basis of this hypothesis are recent kinetic data from our laboratory (37) and others (31-34) that suggest that $OSCN^-$ itself may be too short-lived in physiologic fluids to account for its observed persistence as an antimicrobial agent in a biofilm setting, particularly in the environment of acidogenic biofilms (47, 82, 83). In contrast, we have preliminary data that suggest some of the secondary RSS of the hypothiocyanite system (perhaps O_2SCN^- and O_3SCN^-, by analogy to halide chemistry) are longer-lived and some exhibit reaction properties similar to $OSCN^-$, albeit muted. This suggestion is consistent with the Antimicrobial Depletion (AD) model for the resistance of biofilms toward biocides that react with rates that exceed their rates of diffusion into biofilms (84-86). Referring to Figure 1, one can appreciate a host defense strategy that is adaptive to varied chemical environments.

Possible Roles of the Hypohalite Defensive Mechanisms in the Pathogenesis of Oral Disease?

Gingivitis is associated with maturation of biofilms. Inflammatory response occurs with an increase in plaque mass at the gingival margin, and this in turn results in increased GCF flow. As illustrated in Figure 1, increased GCF flow results in a decrease in the available SCN^- at the gingival margin, which in turn attenuates redox buffering vis-à-vis the reactions of Eqn 3 and 4. As discussed

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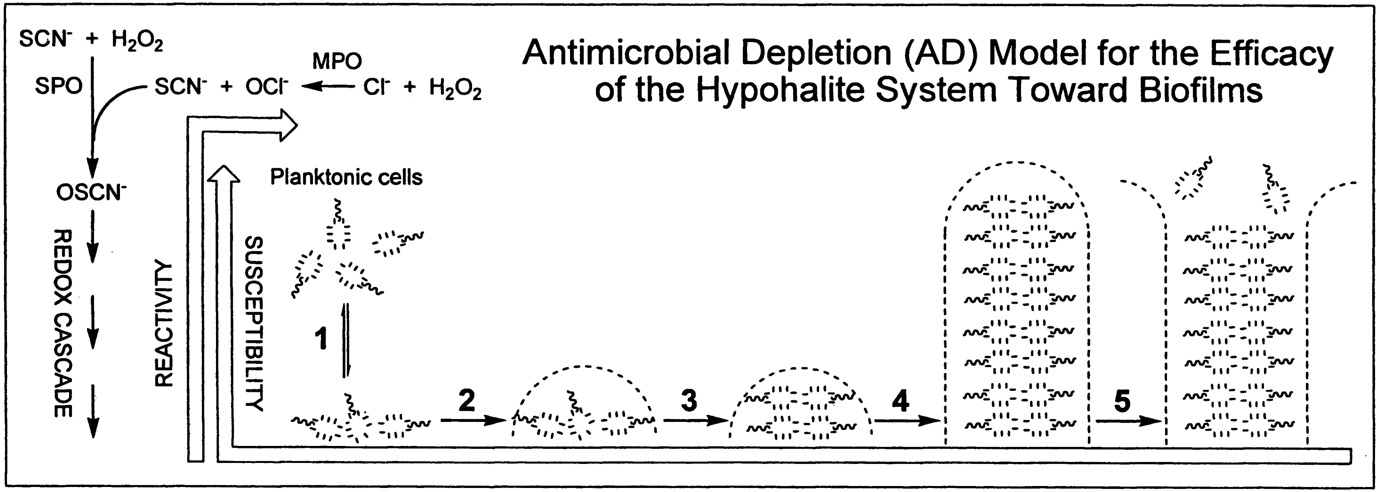
Antimicrobial Depletion (AD) Model for the Efficacy of the Hypohalite System Toward Biofilms
SCN⁻ + H₂O₂
SPO
SCN⁻ + OCl⁻
MPO
Cl⁻ + H₂O₂
OSCN⁻
REDOX CASCADE
REACTIVITY
SUSCEPTIBILITY
Planktonic cells
1
2
3
4
5

Figure 7. Proposed Redox Cascades of the Hypohalites in the Oral Cavity (see text). Hypothetical Stages of Biofilm Maturation: Stage 1 - reversible attachment of cells to the surface. Stage 2 - production of extracellular polymeric substances (EPS) resulting in more firmly adhered "irreversible" attachment. Stage 3 - early development of biofilm architecture. Stage 4 - maturation of biofilm architecture. Stage 5 - dispersion of single cells from the biofilm.

earlier, periodontal disease is associated with a disruption of bacterial homeostasis, presumably as a consequence of environmental pressures. Increased flow of GCF should have the effect of increasing the amount of OCl^- versus $OSCN^-$ that is produced by MPO (Table 1) and decreasing the rate at which OCl^- is sequestered by SCN^- (Eqns 3 and 4). It is interesting to speculate what role, if any, an increase in the production of OCl^- would have on promoting the aforementioned microbial shifts. Unfortunately, very little is known about the efficacies of the hypohalites as biocides toward pathogenic oral bacteria.

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Conclusions

We have presented a progress report of our studies of hypohalites as human defense factors. The oral cavity was chosen as a backdrop for this discussion, in part because it illustrates possible unique roles for the individual hypohalites, but also because the heterogeneous environments that define the oral cavity present a unique challenge for the host defenses. Furthermore, the dynamics of the oral cavity encourage speculation concerning the interplay of chemistry and biology. It is important to recognize that our discussion has focused on the hypohalites, but many other defensive mechanisms are simultaneously operative in the oral cavity. Hence, a challenge exists in establishing cause and effect. It is clear that a better understanding of the impact of the hypohalites on individual oral bacterial strains and the various consortia that form biofilms would facilitate a discussion of the role of hypohalites in health and disease.

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Chapter 13

Peroxygens and Other Forms of Oxygen: Their Use for Effective Cleaning, Disinfection, and Sterilization

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Oxidation may be defined as the process of electron removal, where oxidising agents are substances which cause the loss of electrons from other molecules that are thereby accepted by the agent. As well as their many chemical uses, many oxidizing agents have potent antimicrobial activity. These include various halogens (like chlorine and iodine) and the peroxygens and other forms of oxygen, which include hydrogen peroxide, peracetic acid, chlorine dioxide and ozone. This group of biocides has become widely used for cleaning, antisepsis, disinfection and sterilization applications. This review will discuss the various applications and uses of peroxygens and other forms of oxygen, along with their various advantages and disadvantages. Applications include the use of these biocides in various liquid and gaseous forms, and include food and water disinfection, low temperature surface sterilization and large area remediation, as recently shown with the successful fumigation of buildings following recent bioterrorism attacks in the United States. For liquid applications, the formulation of these biocides plays an important role in optimizing their safety and efficacy. Equally, process controls including the effects of temperature can play an important role in the application of both liquid and gaseous processes. The current understanding of the mode of action of these biocides will be reviewed and recent investigations have identified new applications for these agents in the control of emerging and reemerging pathogens.

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Oxidation may be defined as the loss of an electron by a molecule, atom, or ion and oxidizing agents as agents that remove electrons from the other substances, and are thus reduced themselves. Due to this activity, these agents will have dramatic effects on the structure and function of microorganisms that culminate to give potent biocidal activity. Many oxidizing agents are among the most widely used biocides for medical, dental, industrial and agricultural applications. They can be further classified into the halogens and the peroxygens/other forms of oxygen. The halogens include biocides based on chlorine (like sodium hypochlorite, widely used in bleach solutions), iodine (including iodine-releasing agents such as iodophors) and bromine. The chemical applications and biocidal activities of the halogens have been reviewed elsewhere (1, 2, 3). The peroxygens and other forms of oxygen include relatively simple biocides such as hydrogen peroxide, peracetic acid, chlorine dioxide and ozone. Some of these biocides, in particular hydrogen peroxide, have been used for various antiseptic, preservation and disinfectant applications for over 100 years; yet despite this, many recent advances have been made in the optimization of the antimicrobial activity, surface compatibility and application of these agents both in liquid and gaseous form. The types, uses, and modes of action of the peroxygens and other forms of oxygen are considered further in this review.

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General Mode of Action

The basic mode of action of the peroxygens is to react with the essential macromolecules that make up microbial life, including the oxidation of various proteins, carbohydrates, lipids and nucleic acids (4). These reactions will lead to the loss of structure and function of these molecules, including unfolding, fragmentation and cross-reaction with oxidized groups. Proteins, carbohydrates and lipids on the surface of microorganisms are the initial accessible targets. The various structures on these surfaces are essential to the survival, pathogenicity and basic structure of microorganisms; therefore the loss of structure and function at this stage alone is sufficient to observe loss of viability, in particular as observed in bacterial and viral studies. Initial damage to surface structures is followed by further interactions with various intercellular components, including proteins and nucleic acids, as the structure of the microorganism breaks down. Specific effects of different peroxygen chemicals have been reported as being particularly targeted by these biocides (4). For example, the effects of chlorine dioxide against certain amino acids (tryptophan, cysteine and tyrosine) have been reported. Further, peracetic acid and hydrogen peroxide have been shown to disrupt sulfhydryl (-SH) groups and sulphur bonds (S-S) in proteins, and fatty acid double-bonds; however, it is expected that these groups/bonds will be particularly sensitive to oxidation.

Despite the basic oxidation mode of action of these biocides, the overall effects observed in a microbial population will vary depending on the formulation and delivery process of the biocide. An important aspect in the optimization of the mode of action is access of the oxidizing agent to the microorganism. It is important to note that microorganisms are generally associated with various extraneous materials (or soils) including various proteins, carbohydrates and lipids. If preparations of these microbes in their natural state are added directly to a given concentration of the biocide, clumping may occur which limits the penetration to each microorganism. A clear example is with peracetic acid, simple solutions of which in water have an acidic pH; if a microbial population is exposed to this alone, it will lead to clumping and may underestimate the true activity of the biocide. In contrast, when peracetic acid is provided in a formulation (generally closer to a neutral pH) it can allow greater penetration of the biocide and access to the microorganisms. Overall, unlike anti-infective (including antibiotic) investigations, the formulation and/or process control of a biocide will play a dramatic role in the overall mode of action and optimal activity in microbial inactivation. This is an important, yet often underestimated concept to appreciate. Examples of the impact of these effects will be discussed further.

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Biocidal Applications

Peroxygens are widely used for a range of biocidal applications (Table I). These applications can be considered as being based on the liquid or gaseous biocide at the point of use. Liquid applications can simply use various concentrations of the biocide in water; a notable example is the traditional use of hydrogen peroxide at 3-6% concentrations for skin and particularly wound treatment. In general, most liquid applications use the biocide in various formulations, being in combination with other chemicals that improve the activity, stability, or indeed the surface compatibility of the biocide alone. These formulation chemicals include stabilizing agents, anti-corrosives, surfactants, and chelating agents. For example, peracetic acid is a relatively unstable biocide and is therefore supplied in equilibrium with water, hydrogen peroxide and acetic acid (5). Commercial 35% PAA is provided with 7% hydrogen peroxide, 40% acetic acid and 17% water, which may also contain a stabilizer to improve shelf life. These solutions of peracetic acid can not generally be used directly for disinfection applications as they can be corrosive; however, stable and more compatible formulations based on peracetic acid as the major biocidal agent are widely used for general surface and medical device applications. Similar formulation optimization for antimicrobial efficacy, safety and surface compatibility has been described for hydrogen peroxide, chlorine dioxide, and other mixed oxidants.

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Table I. Biocidal Application with Peroxygens and Other Forms of Oxygen

Applications	*Description*	*Examples*
Preservation	Control of bacterial/fungal growth in a product by inhibition	Limited applications due to reactive and short-lived nature of the biocides. Hydrogen peroxide releasing agents, including benzoyl peroxide
Cleaning	Removal of contamination ('soil') from a surface; can be combined with disinfection	Liquid hydrogen peroxide, peracetic acid and chlorine dioxide
Antisepsis	Microbial reduction or inhibition on living tissues (e.g., skin)	Hydrogen peroxide (including wound treatment)
Water Disinfection	Antimicrobial reduction of viable microorganisms to a safe or defined level in drinking water	Chlorine dioxide (common replacement for chlorine disinfection) Ozone Electrolyzed water
Surface Disinfection (including device and food surfaces)	Antimicrobial reduction of viable microorganisms to a safe or defined level on a surface	Liquid or gaseous hydrogen peroxide Liquid peracetic acid Liquid or gaseous chlorine dioxide Ozone Electrolyzed water
Air disinfection	Antimicrobial reduction of viable microorganisms to a safe or defined level in air	Gaseous hydrogen peroxide and chlorine dioxide Ozone
Sterilization	Defined process to render a surface of product free from viable microorganisms	Gaseous or liquid hydrogen peroxide (including generation as a plasma gas) Liquid peracetic acid (at 50°C) Ozone

Ozone was one of the earlier appreciated gaseous biocides, particularly used for the deodorization and disinfection of air, rooms and some industrial applications. More recently, ozone sterilization systems have been successfully developed for medical device and other surface applications, highlighting advances in ozone generation systems. During the 1980's and 1990's, various applications were developed using gaseous phase peroxygens, including peracetic acid, chlorine dioxide and particularly hydrogen peroxide. These processes have been used for area disinfection, surface (including device) sterilization and other industrial sterilization applications. They include the direct use of the biocide (usually with humidity control, depending on the process) and in combination with an energy source, e.g. in the presence of UV light or the generation of a plasma in the presence of the gas. Plasmas are generated by energizing the molecules of a gas to give a highly excited mixture of charged, reactive nuclei and free electrons. Plasma-based disinfection and sterilization systems have been described with peracetic acid, oxygen (for ozone generation) and notably hydrogen peroxide.

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Peracetic Acid

Peracetic acid (CH_3COOOH; PAA) is a potent biocide at relatively low concentrations and is also considered environmentally friendly as it rapidly breaks down to water, oxygen and a low concentration of acetic acid (5). Peracetic acid is also unique as an oxidizing agent in that it demonstrates somewhat stable activity even in the presence of organic and inorganic soils, which can interfere with optimal activity. Its main applications have been in liquid formulation disinfectants, in particular to optimize compatibility concerns with a broad spectrum of plastics, metals and other surface materials, as well as improved biocide stability. It should be noted that these formulations can vary significantly in antimicrobial efficacy and surface compatibility, independent of the concentration of peracetic used in the formulation. With optimized formulation, peracetic acid solutions can be successfully used on many sensitive materials, including those used in flexible endoscopes and dialysis equipment (5). Peracetic acid formulations are used for cleaning (to physically remove and breakdown soils), surface disinfection, waste-treatment and device disinfection/sterilization. Direct use of concentrated peracetic acid is limited to certain industrial applications, but formulations include stabilized solutions, two-component solutions (one containing peracetic acid and the other with formulation excipients) and generational type formulations. An example of a generational type reaction to make peracetic acid in formulation is shown in Figure 1.

Gaseous peracetic acid disinfection (fumigation) and sterilization systems have also been described but have not seen widespread use due to the aggressive nature of the biocide in vapor form on various surfaces.

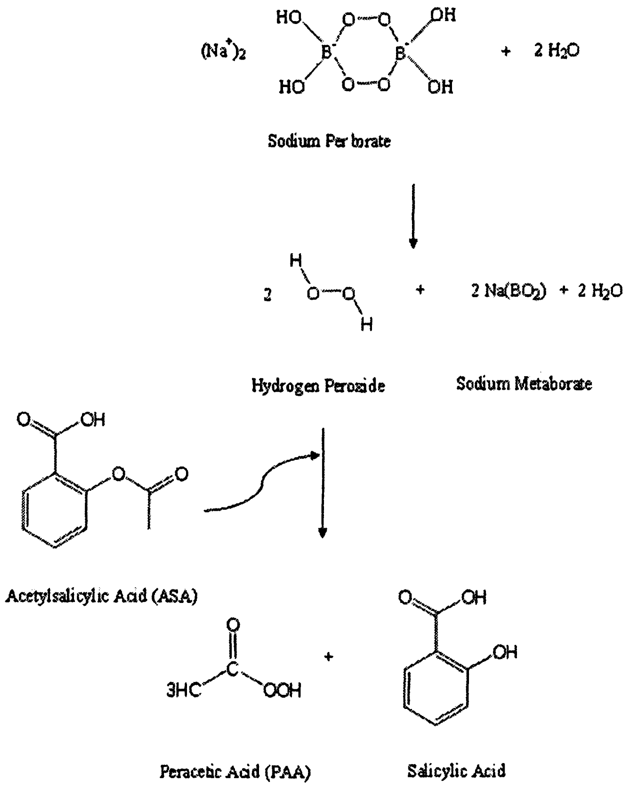

Figure 1. An example of a peracetic acid generational chemistry from sodium perborate and acetylsalicylic acid.

A unique low-temperature liquid chemical sterilization system, the STERIS SYSTEM 1, was one of the first peracetic acid-based systems designed for the reprocessing of thermosensitive medical and dental devices including flexible endoscopes (6). The system consists of a processing unit that dilutes and flows a peracetic acid-based formulation. This is delivered as a two-component chemistry in a cup-within a-cup design (STERIS 20) consisting of ~35% peracetic acid solution, separated from the dry formulation components that are dissolved, mixed and heated during the sterilization process. In the final sterilization process, the concentration of peracetic acid is ~0.2%, at a pH of 6.4 and at 50-55°C. The total process time is ~30minutes, consisting of a 12 minute chemistry contact, followed by four sterile water rinses; this process has been shown to comply with the efficacy requirements of ISO 14937 as a true sterilization process (6, 7). The broad spectrum antimicrobial efficacy of the process is due to a combination of peracetic acid, the formulation and contact temperature to include bacteria, molds, yeasts, bacterial and fungal spores, viruses and protozoal dormant form (cysts/oocysts). Recent studies on the mode

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of action of action of this process highlight the importance of formulation and process control in the activity of peracetic acid. For example, the process has been shown to cause the degradation of proteins, but only within the range of 45-60°C (Figure 2); at higher temperatures, for example, potential clumping of test proteins has been observed that may be expected due to the effects of temperature alone on protein structure.

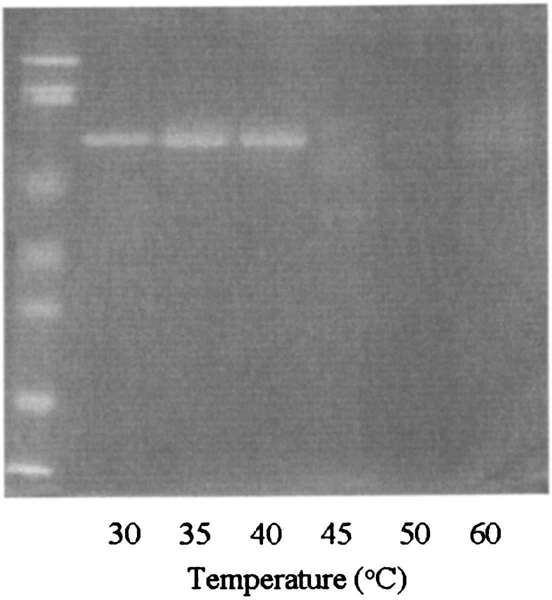

Figure 2. SDS-PAGE analysis on protein (bovine serum albumin) samples exposed to the STERIS 20 formulation at various contact temperatures.

These effects on the structure of proteins may be significant in the control of 'infectious' proteins (known as prions) that have been implicated as the causative agents in a rare group of diseases known as transmissible spongiform encephalopathies (TSE's) such as Creutzfeld-Jakob disease (CJD) and Bovine Spongiform Encephalopathy (BSE). The prion proteins are proposed to transmit these diseases in the absence of any known nucleic acid and are known to be intrinsically resistant to disinfection and sterilization methods (8). Biocides that have a cross-linking mode of action such as glutaraldehyde and formaldehyde are not recommended to treat these agents, but some studies have shown promising efficacy with alternative oxidizing agent formulations and processes (9). Peracetic acid in the STERIS 20 formulation has demonstrated some activity to degrade prion proteins, but only in this formulation and when tested at 50°C. The mode of action would appear to be a combination of the exposure temperature

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and formulation excipients that may allow for partial unfolding of the protein, and the ability of peracetic acid to degrade the protein structure. pH could also be an important variable, as when protein is added directly to a solution of peracetic acid it will cause clumping and, therefore, lack of biocide penentration; in contrast, peracetic acid in formulation under controlled pH conditions can show effective protein degradation.. Other formulations and uses of peracetic acid require further investigations and can not be assumed to have any efficacy against prions due to their unique structure and infectivity profile (e.g., as shown under experimental conditions, 10).

Similar effects of formulation and process control in the activity of peracetic acid have been described in biofilm disinfection investigations. Biofilms are communities of microorganisms (either singular or multiple species) that develop on or are associated with surfaces (11). As biofilms grow and mature, they are found within an external matrix of protein, carbohydrate and other materials that can provide an excellent protection (and therefore resistance) mechanism to biocides and biocidal processes (4). The presence of biofilms has been implicated in microbial survival (e.g., in nosocomial infections with the use of inadequately reprocessed medical devices, product spoilage and cross-contamination) as well as surface damage over time. For these reasons, biofilm control is an important consideration and in particular in the disinfection of water and water-based systems. Peracetic acid-based formulations have often been recommended over aldehyde disinfectants as they have been proposed to better penetrate and remove biofilms from surfaces. This in fact may not always be the case, as exemplified by recent studies by Martiny *et al.* (10). In this report, the activity of two peracetic acid-based disinfectants to decontaminate surfaces was compared to aldehyde-based disinfectants. Although the aldehydes showed an expected lack of activity, the peracetic acid-based products also showed clumping of the biofilm on surfaces, thereby limiting penetration, removal and disinfection. In contrast, the STERIS 20 process has been shown to degrade and remove *Pseudomonas aeruginosa* biofilm proteins and carbohydrates from contaminated surfaces, allowing adequate disinfection of intrinsic microorganisms (as shown in Figure 3). These results highlight the importance of formulation and process control in the activity of peracetic acid on surface disinfection and overall mode of action of the biocide.

Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a simple yet powerful oxidizing agent and biocide (12). It may also be considered environmentally friendly, rapidly degrading into water and oxygen. Liquid applications include direct use on the skin (as described above) or for industrial applications, and in formulation, in particular to enhance the stability and antimicrobial activity at lower concentrations. It has also been widely used as an effective biocidal gas (13).

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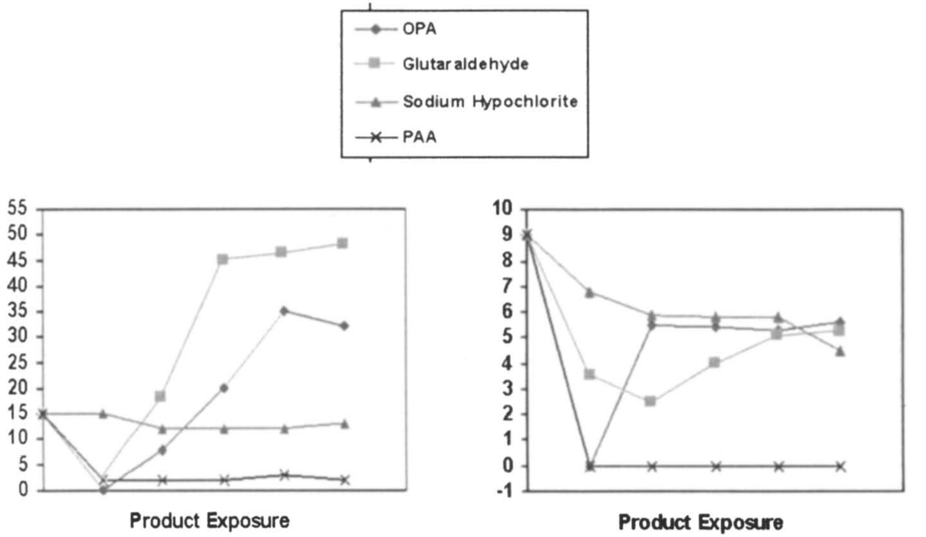

Carbohydrate Concentration
(µg/cm^2)

Bacterial Count
(log$_{10}$ cfu/cm^2 *Pseudomonas aeruginosa*)

Figure 3. Results of a biofilm investigation with 4 disinfectants: 0.2% peracetic acid (STERIS 20 at 50°C), 0.5% ortho-phthaldehyde, 2% glutaraldehyde and 10% bleach (~0.5% sodium hypochlorite). Pseudomonas aeruginosa biofilms were developed on test surfaces and exposed daily to individual disinfectant treatments. The levels of carbohydrate, protein (not shown) and viable bacteria were evaluated. The first point (at the y-axis of both graphs) indicates initial, unexposed levels of bacteria or carbohydrate detected and subsequent points are following exposure (up to 5) to the test disinfectants. Resistant, stable biofilms developed over repeated applications of all biocides, with the exception of the exposure to the peracetic acid formulation.

As for other oxidizing agents, the overall surface compatibility and antimicrobial efficacy will be formulation dependant (in the case of liquid applications) and process-dependant. An example is in the use of gaseous hydrogen peroxide; in its true gaseous or vapor form, peroxide demonstrates more potent antimicrobial activity that at similar concentrations in liquid form (e.g., ~350mg/L of liquid to give similar activity to 1-2mg/L of the gas), but also greater compatibility with various surface materials including electronics and other sensitive materials. However, with consideration to the physical chemistry of the biocide, if the concentration of gaseous peroxide is increased above a given saturation point (dependant on the temperature) it will condense out on surfaces at high liquid concentrations (~70-80%) and pose additional safety and compatibility concerns than the gas.

Hydrogen peroxide is used as an effective antiseptic, wound cleaner, hard surface cleaner, surface disinfectant and sterilization applications (both in liquid and gaseous forms; 4, 12). As an example, various gaseous applications with hydrogen peroxide are discussed in further detail. Hydrogen peroxide gas is readily generated by vaporization of liquid peroxide solutions (generally 30-60%) at 100°C (or at lower temperatures under vacuum), to give a colorless, odorless gas. The broad spectrum activity of the gaseous form has been well studied and published including bactericidal, fungicidal, viricidal, sporicidal, cysticidal, oocidal and, more recently priocidal activity (9, 13). The antimicrobial activity of gaseous peroxide is unique in that it is effective at both high and low humidity levels levels, where in contrast >60% humidity is required for other biocides like ozone, chlorine dioxide, formaldehyde and ethylene oxide. Gaseous processes include the fumigation of enclosed spaces such as isolators, laminar flow cabinets, rooms, vehicles and buildings. For example, peroxide gas was successfully used in the remediation of buildings contaminated during the anthrax spore bioterrorism events in the United States during 2001. Successful fumigation applications have been subsequently described in other general, industrial, and medical (hospital) applications (13, 14). The overall process is similar in all these applications using gas generator systems to dehumidify the given area to ~40% relative humidity, conditioning the area to a given concentration of peroxide (~0.1-2mg/L), decontamination (by maintaining the biocide concentration) and aeration to remove peroxide gas to a safe level (~1ppm; 13). In addition to antimicrobial activity, gaseous peroxide has been shown to effectively neutralize bacterial exotoxins (including the protein-based anthrax and botulinum bacterial toxins), some chemical weapons and some cytotoxic drugs (15) for other applications. Sterilization processes have been developed for industrial, food packaging, medical and dental applications. These generally are performed under vacuum to optimize gas delivery, penetration and safe removal during a given process. Examples of gaseous sterilization apparatus are shown in Figure 4 and include processes with and without plasma generation as part of the sterilization process. Other sterilization processes control the temperature and flow of the gas to ensure adequate surface contact for the required exposure conditions.

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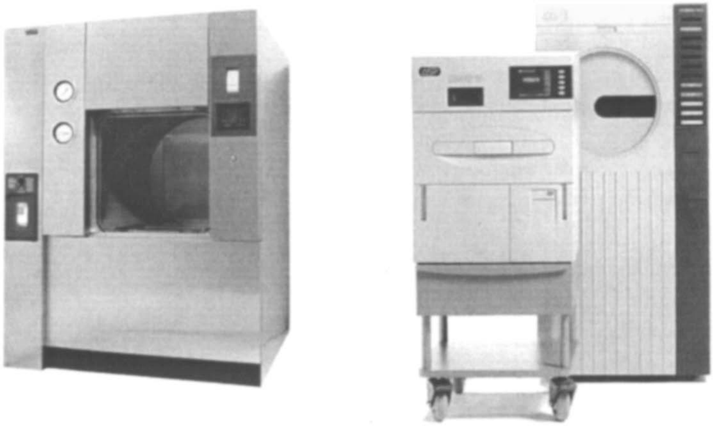

Figure 4. Hydrogen peroxide gas sterilization equipment. On the left is the STERIS VHP MD system for medical device sterilization and on the right are the STERRAD NX (middle) and 100S systems for reusable medical device sterilization in hospitals. The STERIS system uses VHP under vacuum and the STERRAD process uses condensed peroxide gas in combination with plasma generation.

The mode of action of hydrogen peroxide is based on both the peroxide molecule itself, as an effective oxidizing agent, as well as other short-lived radicals and ions (e.g., ${}^{\bullet}HO$, O^*, ${}^1O_2$, $HOO^{\bullet}$) that form during the degradation to water and oxygen (4). It may be expected that the increased reactivity of the peroxide molecule and greater presence of break-down species may be responsible for the increased activity of gaseous over liquid peroxide. These various species will rapidly react and oxidize various groups on external/internal microbial constituents including lipids, proteins and nucleic acids. Some of these effects at a molecular level have been described in detail, as hydrogen peroxide is a natural by-product of the respiration of eukaryotic and prokaryotic cells, and unless controlled can cause internal damage to cell components by structure disruption and loss of function (16). Hydrogen peroxide gas has been shown to react with and disrupt peptide bonds, leading to protein fragmentation. This attribute was subsequently investigated for activity against prion proteins and was shown to be an effective priocidal biocide (9); interestingly, in contrast it was also shown that the activity of liquid (or condensed) peroxide against proteins and prions was dramatically limited (Figure 5). In studies with prion protein preparations, gaseous hydrogen peroxide was shown to degrade the prion proteins, while exposure to liquid peroxide caused clumping, lack of biocide penetration and little to no activity. It may be expected that similar clumping may also occur and prevent biocidal penetration when microorganisms are surrounded by contaminating soils, limiting the activity of liquid peroxide. It is certainly conceivable that these effects may be limited or activity enhanced by liquid formulation effects similar to those described for peracetic acid.

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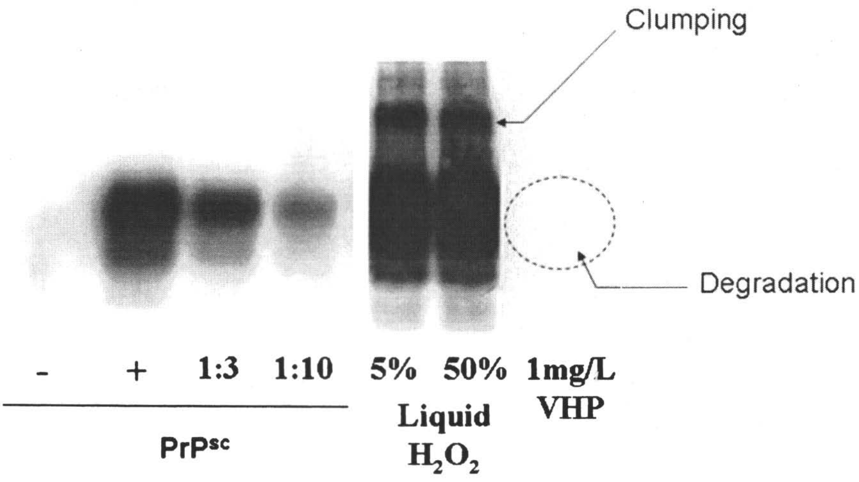

Figure 5. The activity of hydrogen peroxide against prion proteins. This Western blot demonstrates dilutions of a prion protein (PrP^{sc}) preparation on the left and the effect of immersion in liquid hydrogen peroxide at 5 and 50% v/v in water and on exposure to vaporized hydrogen peroxide (VHP).

Although the subtle effects of low concentrations of hydrogen peroxide on nucleic acids have been described to include reactions with nucleotide bases and the sugar-phosphate backbone, the effects at truly biocidal concentrations have only recently been investigated. Exposures to gaseous hydrogen peroxide have shown significant nucleic acid unfolding and fragmentation, depending on the gas concentration and exposure time (McDonnell, unpublished results). These effects will obviously initially disrupt the essential replication, transcription and translation activities of nucleic acids, but will also eventually culminate in enough damage that any natural repair mechanisms of the cell will not allow for recovery. The inactivation of nucleic acids in a important attribute for any biocide or biocidal process, in particular in consideration of viricidal activity, where the viral nucleic acid has in some cases been shown to be able to infect cells despite loss of viral structure (17).

Chlorine Dioxide

Chlorine dioxide (ClO_2) is an unstable, water soluble gas (18). It is used as an effective biocide in various liquid and gaseous applications; however, due to its intrinsic instability, it needs to be generated at its site of use by various different chemical and electrochemical methods. Examples of various chemical reactions used in the generation of chlorine dioxide are shown in Figure 6.

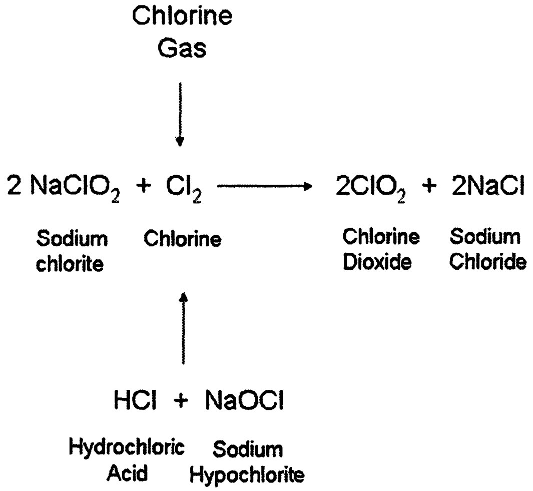

Figure 6. Examples of the generation of chlorine dioxide by various chemical reactions, via the reaction of chlorine with sodium chlorite.

The biocide is also considered environmentally friendly, as it rapidly breaks down into innocuous components. Chlorine dioxide is directly used in liquids (including water), in formulation (usually as two-components which are mixed to generate the biocide in the presence of other formulation excipients) and in gaseous form (in the presence of >70% relative humidity; 18). These applications all demonstrate broad spectrum antimicrobial activity over recommended product/process contact times and conditions. Surface compatibility is generally recorded as being less than that experienced with hydrogen peroxide (depending on the process control, formulation and biocide concentration); despite this, gaseous and liquid applications have been successfully developed for disinfection of even sensitive plastics used in reusuable, thermosensitive devices or other surfaces. In gaseous applications, incompatibility observed with surfaces has been linked to the production of chlorine-intermediates that can particularly form on degradation on exposure to light (thereby fumigation applications are best applied under darkness). Chlorine dioxide has been particularly widely used for water disinfection (as a safer alternative to the use of direct chlorination) and food surface disinfection, with other applications including general surface disinfection (in liquid and gas phases) and, in gaseous applications, for use in medical/dental sterilization processes (although many of these systems are not currently commercially available). Antiseptic applications, at lower concentrations have also been used in limited situations. Various two-component formulations are currently used for device disinfection and gaseous chlorine dioxide has been successfully

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applied for large area fumigation, notably for building decontamination in the remediation of anthrax spore contamination in the United States.

Other Oxygenated Species

Ozone (O_3) is probably the most reactive and short-lived oxidizing agent used for biocidal applications (19). It may be produced by passing oxygen (or the oxygen in air) through a high energy source (e.g., UV light, electrochemical cells or corona discharge). Ozone itself is a simple molecule, which rapidly degrades on reaction with surfaces, contaminants etc. into water and oxygen. The reactivity of the molecule makes it a very effective antimicrobial in both liquid and gaseous applications (the latter requiring humidity control of generally >70% relative humidity). In addition, during the generation and degradation of ozone, many other oxygenated species are produced including ions (e.g., the superoxide O^{2-} and peroxide O_2^{2-} ions) and radicals (hydroxyl $^{\bullet}OH$ and hydroperoxyl HO_2 radicals); these are equally reactive and will culminate in the overall antimicrobial activity associated with ozone. However, it is also the reactivity of ozone and other species that can cause some of the disadvantages in the use of the biocide, in particular surface compatibility (e.g., various plastics and metals) and maintenance of microbiocidal concentrations over the required exposure times (especially for endosporicidal activity).

Ozone is used for water disinfection, deodorization, surface disinfection (in particular in gaseous form) and as a gaseous sterilization process. The use of ozone as a biocide has been advanced by the development of more efficient and reproducible ozone generation systems. For example, although various patents and processes have been described, it is only recently that ozone sterilization for devices has become more widely available (Figure 7; 20).

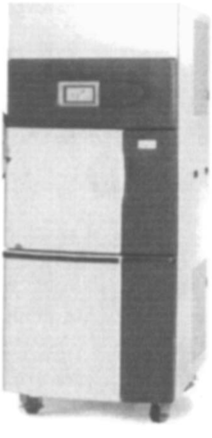

Figure 7. The TSO3 125L ozone sterilizer.

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This new sterilization process uses a >3 hour exposure time including humidification of the load (at 85-95% relative humidity), ozone exposure and ventilation. Although being a simple, economic and effective sterilization method, the process is currently limited due to some material restrictions and compatibility concerns over multiple exposure times. Ozone itself has been well studied as an antimicrobial, but initial claims of some priocidal activity have yet to be substantiated and are under further investigation.

Electrolyzed water generators use water and a low concentration of sodium chloride (or another salt) to generate a solution of mixed oxygenated species by passing solutions through an electrolysis device, subjecting it to a voltage across a membrane and collection of the resulting anolyte for direct use as an antimicrobial solution (Figure 8). The parallel collected catholyte solution has also been used as an effective cleaning solution.

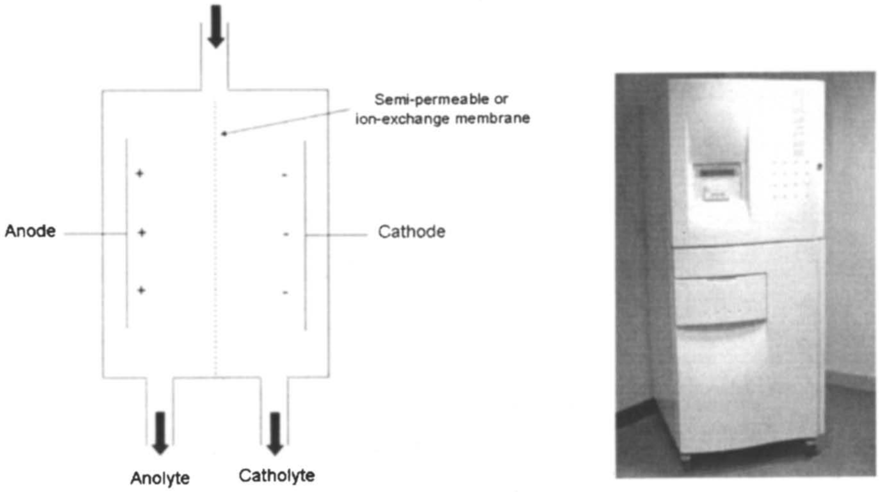

Figure 8. Electrolyzed water generators. The theory of operation is shown on the left, by passing the water sample through an electrolysis unit and separation of the anolyte which is used for biocidal purposes. An example of a STERILOX generator is shown on the right.

The biocidal activity of the anolyte is primarily due to the generation of hypochlorous acid (HOCl), but also other oxygenated species such as ozone and superoxide radicals (21). A variety of systems have been developed, some of which control the pH of the resulting anolyte (which is acidic) by mixing with a

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given proportion of the basic catholyte portion also separated during generation. The anolyte is rapidly antimicrobial, but is also short-lived (thereby requiring to be generated at site of use) and environmentally friendly. Similar to ozone applications, although having the advantage of potent biocidal activity, the active species are quickly neutralized in the presence of contaminating soils and can also be rather aggressive to sensitive surface materials. For example, even in pH adjusted systems, a specialized protective coating is recommended to be applied on flexible endoscopes being reprocessed with electrolysed water to prevent damage on repeated uses. Overall, successful applications of these systems have included odor control, water disinfection and low temperature device disinfection.

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Conclusions

Peroxygens and other forms of oxygen are widely used for cleaning, antiseptic, disinfection and sterilization applications. They have been shown to be potent biocidal agents, including activity against those standard investigated microorganisms such as bacteria, fungi and viruses, as well as less studied pathogens such as parasites and prions. Biocidal activity can be successfully and safely applied on a variety of surfaces with little overall environmental impact. However, it is clear, yet often underestimated, that the success of these requirements will depend of the formulation of the biocide (in liquid applications) and in the control of liquid/gaseous processes (e.g., temperature, delivery, humidity etc.). What may appear to be similar formulations and processes may indeed pose varying antimicrobial activities, surface compatibility profiles and safety considerations. These variables should not be underestimated in the application and use of peroxygens for biocidal purposes. Further, these effects will also play a role in the overall mechanisms of action of these biocides. Peroxygens, as oxidizing agents, clearly have non-specific modes of action against the various macromolecules that make up microbial life. The oxidation of these molecules causes a loss of essential structure and function, which culminate in a loss of viability; however, the various formulation and process effects associated with these biocides have been shown to play an important role in the optimization and even restriction of these effects. It is clear that in the future that further improved formulations, delivery/control processes and optimized applications will be developed with peroxygens and other oxygenated species, as well as a greater understanding of their spectrum and mechanisms of biocidal activity.

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Chapter 14

Electrolyzed Water: Principles and Applications

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Electrolyzed oxidizing (EO) water produced though salt water electrolysis with strong antimicrobial properties was recently introduced as a new sanitizer for the food industries. Studies have shown that EO water is effective on inactivating pathogens including *Escherichia coli* O157:H7, *Salmonella enteritidis*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Enterobacter aerogenes*, and *Staphylococcus aureus*. Applications of EO water as a disinfectant for reducing microbial contamination have been reported for vegetables, fruits, eggs, poultry, seafood, cutting boards, and food contact surfaces. This Chapter provides an overview of the chemistry and antimicrobial properties of EO water and potential applications of EO water as an alternative to chlorine-based compounds for inactivating bacteria on raw materials and food-processing surfaces.

Electrolyzed water, produced through electrolysis of a dilute salt solution (0.05-0.2% NaCl), was first reported in the 1970s to possess antimicrobial properties (*1*). During water electrolysis, two types (alkaline and acidic) of waters are produced from each electrode side with either high reducing (alkaline water) or high oxidizing (acidic water) potential. The electrolyzed reducing (ER)

In New Biocides Development; Zhu, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

water has a high pH of about 11.4 and the electrolyzed oxidizing (EO) water has a low pH of about 2.5. The EO water contains hypochlorous acid with a low pH value and a high oxidation-reduction potential (ORP), and has been reported to exhibit strong antibacterial activities against a variety of microorganisms including many foodborne pathogens. It was recently introduced as a new anti-microbial agent and can be used as a chlorine alternative for reducing microbial contamination on raw materials and food processing surfaces.

Water Electrolysis

The generation of EO water involves reactions in a cell containing inert positively and negatively charged electrodes separated by a membrane through which a dilute salt solution is passed (Figure 1). By subjecting the electrodes to a DC voltage of about 8 to 10 volts, two types of water possessing different characteristics are generated: (1) electrolyzed basic solution with high reducing potential, which can reduce free radicals in biological systems and (2) electrolyzed acidic solution with high oxidation potential and strong bactericidal property. Clinical studies have demonstrated that the electrolyzed basic solution could destroy free radicals and reduce oxidation of highly unsaturated fats and oils in biological systems (*2*) and the electrolyzed acidic solution was effective on inactivating various types of infectious microorganisms including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Bacillus cereus*, *Campylobacter jejuni*, and *Salmonella enteritidis* (*3, 4, 5, 6*).

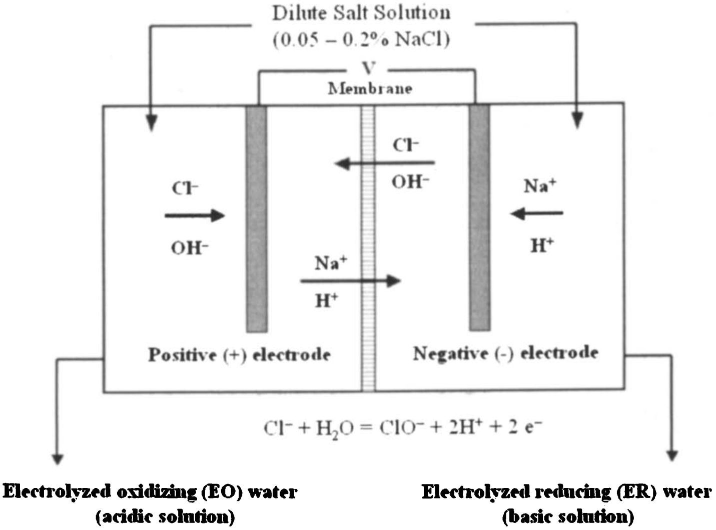
Dilute Salt Solution
(0.05 – 0.2% NaCl)
V
Membrane
Cl^-
OH^-
Cl^-
OH^-
Na^+
H^+
Na^+
H^+
Positive (+) electrode
Negative (-) electrode
$Cl^- + H_2O = ClO^- + 2H^+ + 2\,e^-$
Electrolyzed oxidizing (EO) water
(acidic solution)
Electrolyzed reducing (ER) water
(basic solution)

Figure 1. Electrolyzed water production.

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Electrolyzed Oxidizing (EO) Water

General properties of EO water include low pH (ca. 2.5), high ORP (ca. +1,150 mV) and hypochlorous acid. The properties of EO water can be adjusted by changing processing parameters, such as salt concentration, voltage, and electrolyte flow rate used in water electrolysis. Studies conducted by Ezeike and Hung (*7*) using response surface methodology (RSM) found that NaCl concentration was the most significant factor affecting pH, ORP, and residual chlorine content of EO water. Besides the salt concentration, electrolyte flow rates and voltages can also be critical parameters affecting properties of EO water. In general, the higher the NaCl concentrations and voltages, the higher the ORP and residual chlorine will be produced in EO water. However, in the case of residual chlorine, flow rate was relatively more important than voltage. Increasing electrolyte flow rate resulted in lower ORP and residual chlorine in EO water due to reduced residence time of salt water in the electrolytic cell.

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Antimicrobial Properties of EO Water

The bactericidal effect of EO water has been reported to relate to its available chlorine, high ORP, and low pH. Studies conducted by Kim *et al.* (*8*) suggested that the ORP played a primary role in the disinfection capability of EO water, while other studies reported that the available chlorine (Cl_2, HOCl, OCl^-) in EO water was the main component for its antimicrobial activity (*9, 10*).

Hypochlorous Acid

Chlorine is the most widely used sanitizer and is known to be most active in its hypochlorous acid form. Hypochlorous acid (HOCl) is produced during the salt water electrolysis and plays an important role in EO water's antibacterial activities. At its neutral charge, HOCl can penetrate the bacterial cell wall and interact with key metabolic key enzymes to prevent normal respiration (*11*). Therefore, increasing salt concentrations in water for electrolysis usually results in increased amounts of HOCl in EO water with stronger antibacterial activities. Study conducted by Len *et al.* (*12*) found a strong correlation ($r = 0.95$) between concentrations of hypochlorous acid (HOCl) and the antibacterial activity of EO water, suggesting that HOCl was the primary antibacterial agent in EO water. Park *et al.* (*13*) studied the effects of chlorine and pH of EO water on inactivating *E. coli* O157:H7 and *L. monocytogenes* and found that the bactericidal activity of EO water increased with residual chlorine concentration and complete inactivation (about 7.0 log CFU/ml reduction) of either pathogen was achieved at residual chlorine levels of ≥1.0 ppm. A study conducted by Liu *et al.* (*14*) also reported that the bactericidal effect of EO water against *L. monocytogenes* was positively correlated with chlorine contents in EO water.

The concentrations of hypochlorous acid in EO water can be affected by chloride ion (Cl^-) in the water. Free chlorine gas can be formed through interaction between HOCl and Cl^- and loss of chlorine gas will result in reduced concentrations of HOCl and bactericidal effects of EO water.

$$HOCl + Cl^- + H^+ \Leftrightarrow H_2O + Cl_2$$

Under open conditions, the loss of chlorine through evaporation was reported to follow first-order kinetics. The rate of chlorine loss was increased by about 5-fold with agitation, but was not significantly affected by diffused light (*15*). On the other hand, the chlorine loss did not follow first-order kinetics under closed conditions because the primary mechanism of the chlorine loss might be related to disproportionation of chlorine rather than evaporation. Therefore, EO water should be used right after production or be kept in a closed container before use to allow the exhibition of maximal antimicrobial activity.

Oxidation-Reduction Potential (ORP)

The high ORP value is an important factor contributing to the antibacterial activity of EO water. Most aerobic bacteria require a positive ORP (+200 to +800 mV) while anaerobic bacteria need a negative ORP (-40 and -400 mV) to grow. The high ORP (ca. 1,150 mV) of EO water creates an environment that is not suitable for growth of bacteria. Kim *et al.* (*8*) compared the pH, ORP, and residual chlorine of EO water for inactivating *E. coli* O157:H7 and found that both ORP and residual chlorine contributed to bacterial inactivation and suggested that the high ORP might be the primary property responsible for EO water's bactericidal effect.

A similar study was conducted by Liu *et al.* (*14*) to compare the antibacterial activities of acidity, ORP, and residual chlorine of EO water against *L. monocytogenes* on stainless steel surface. In the study, *L. monocytogenes* was inoculated to stainless steel chips (5×5 cm^2) and soaked in tap water, EO water with reduced ORP value (modified EO water), or EO water for 5 min. Results showed that soaking inoculated chips in modified EO water (pH:2.51, ORP:561 mV, chlorine:40 ppm) resulted in a small degree reduction of *L. monocytogenes* (1.55 log CFU/chip) while a much greater reduction of *L. monocytogenes* (4.23 log CFU/chip) was yielded by a treatment of chlorine water (pH:3.43, ORP:1033 mV, chlorine:40 ppm) containing same chlorine content but a higher ORP value (Table 1). A treatment with EO water (pH:2.65, ORP:1155 mV, chlorine:40 ppm) containing ORP higher than that of chlorine water completely inactivated *L. monocytogenes* on the stainless steel surface

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(>4.76 log CFU/chip). These results confirm that the high ORP plays an important role in EO water's antibacterial property.

Table 1. Bactericidal effects of pH, ORP and chlorine of EO water on *L. monocytogenes* on stainless steel surface.

Treatments	*pH*	*ORP (mV)*	*Chlorine (ppm)*	*L. monocytogenes* [a]
No treatment				5.76 ± 0.23 A
Tap water	6.85	541	0	4.96 ± 0.09 B
Acidic water	2.65	630	0	4.35 ± 0.14 C
Modified EO water	2.51	561	40	4.21 ± 0.26 C
Chlorine water	3.43	1033	40	1.53 ± 0.17 D
EO water	2.65	1155	40	ND [b] E

[a] Mean (Log CFU/chip) of triplicate determinations ± standard deviation. Means with the same letter in the same column are not significantly different ($p > 0.05$).

[b] Not detectable by plate count method with a detection limit of <10 CFU/chip.

SOURCE: Data are from reference (*14*).

pH

Most bacteria, except a few such as lactic acid bacteria, can only grow in limited pH range between 4 and 9. The pH of EO water is usually around 2.5 and, therefore, has inhibitory effect on growth of many bacteria. However, the low pH has only limited bactericidal effects. Park *et al.* (*13*) reported that bactericidal activity of EO water increased with decreasing pH. However, with sufficient residual chlorine (>2 ppm), EO water can be applied in a pH range between 2.6 and 7.0 while still achieving complete inactivation of *E. coli* O157:H7 and *L. monocytogenes*. Studies conducted by Liu *et al.* (*14*) reported that a treatment of EO water for 5 min reduced *L. monocytogenes* on clean stainless steel surface by >4.7 log CFU/chip, while a treatment of acidic water with same pH of EO water but much lower ORP and no chlorine could only reduce populations of *L. monocytogenes* by about 1.4 log CFU/chip (Table 1). Although the study indicated that both chlorine and high ORP value are more important than low pH in contributing to EO water's antibacterial activity, it was very clear that EO water could not exhibit the strongest bactericidal effects without available chlorine, high ORP, and low pH value.

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Antibacterial Activity of EO Water

Extensive research on EO water has been conducted at the University of Georgia to evaluate the efficacy of EO water to inactivate foodborne pathogens including *E. coli* O157:H7, *L. monocytogenes*, *Salmonella*, and *Bacillus cereus*. Venkitanarayanan *et al.* (*4*) found that an exposure time of 5 min at 4 or 23°C in EO water (43-86 ppm free chlorine) resulted in approximately 7-log (CFU/ml) reductions of *E. coli* O157:H7, *Salmonella enteritidis*, and *L. monocytogenes*. Kim *et al.* (*5*) reported that a treatment of EO water (pH:2.6, ORP:1,160 mV, chlorine:56 ppm) for 10 s reduced populations of *E. coli* O157:H7 and *L. monocytogenes* by >8.6 log CFU/ml. *B. cereus* was found more resistant than *E. coli* O157:H7 or *L. monocytogenes* to the EO water treatment and required a 30 s treatment for a complete inactivation (>6.6 log CFU/ml) of vegetative cells. Spores of *B. cereus* were found to be much more resistant than the vegetative cells. However, approximately 3.5-log reduction of *B. cereus* spore was achieved with 120 s of the EO water (56 ppm chlorine) treatment. Liu *et al.* (*14*) also reported strong bactericidal effects of EO water against *L. monocytogenes*. Populations of a five-strain cocktail of *L. monocytogenes* decreased rapidly from 9.5 to <1.0 log CFU/ml in EO water containing 50 ppm chlorine within 10 s at room temperature.

Application of EO Water as Disinfecting Agent

Recently, EO water has received great attention in the food industries as an alternative to chlorine for reducing microbial contamination. Many studies have demonstrated strong antibacterial activity of EO water against foodborne pathogens on raw materials and food contact surfaces. Application of EO water as a disinfectant for reducing microbial contaminations has been reported for fresh fruits and vegetables (*3, 16, 17, 18, 19, 20, 21*), poultry carcass (*6, 22*) and shell egg (*23*), cutting boards (*24*), and food processing surfaces (*14, 25, 26*).

Vegetables

Fresh fruit and vegetables may contain numerous microorganisms including human pathogens (*27*). Although washing fruits and vegetables with clean water is the most commonly used method to reduce bacterial contamination, studies have shown that washing raw produces with water had limited effects on reducing total bacterial populations on the surfaces (*28, 29, 30*). However, studies have shown that bacterial contamination on fresh fruits and vegetables

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could be effectively reduced by washing with electrolyzed water. Koseki *et al.* (*16*) reported that washing lettuce in electrolyzed reducing (ER) water for 1 min followed by in EO water for 1 min reduced viable aerobes by 2 log CFU/g without damaging surface structure of lettuce. Park *et al.* (*17*) also reported that washing lettuce leaf with EO water for 3 min significantly ($p<0.05$) reduced population of inoculated *E. coli* O517:H7 and *L. monocytogenes* by 2.41 and 2.65 log CFU/g, respectively, without creating significant changes in quality of washed lettuce during 2 weeks of storage. In addition, the bactericidal effect of EO water was also observed in ice form. Populations of aerobic bacteria on lettuce was reduced by 1.5 log CFU/g when lettuce was stored in ice made of EO water and kept at 2-3°C for 24 h (*19, 31*).

Recently, studies have been conducted to determine the combined effects of ER and EO waters on reducing bacterial contamination. Although a practical use of ER water as a sole washing or sanitizing agent has not yet been developed, studies have found that ER water could be used as a pre-wash agent and was applicable for a combined usage with EO water to enhance microbial reduction in vegetables (*21*). Washing lettuce inoculated with *E. coli* O157:H7 and *Salmonella* with mildly heated (50°C) ER water for 5 min followed by a wash with chilled (4°C) EO water for 1 min could lead to 3-4 log CFU/g reductions of both *E. coli* O517:H7 and *Salmonella* on the lettuce. The study also found that extending the time of heated ER water pre-treatment resulted in greater bactericidal effect than by extending the time of subsequent wash with chilled EO water. The combined electrolyzed water treatment was reported to have no negative effects on appearance of treated lettuce.

In addition to lettuce, studies have also been conducted to determine the bactericidal effects of EO water on reducing bacterial contamination on fresh cucumber and alfalfa sprouts. Total aerobic mesophiles naturally occurring on cucumber were reduced by 1.4 log CFU per cucumber after being treated with EO water (30 ppm chlorine) for 10 min (*20*). The reduction in bacterial population was significantly ($p<0.05$) greater than the reduction (0.7-log CFU per cucumber) achieved with an ozonated water (5 ppm ozone) treatment. The reduction of bacterial populations on cucumber was increased to >2.0 log CFU per cucumber when cucumber was first washed in ER water for 5 min and then in EO water for 5 min. Kim *et al.* (*18*) found that application of EO water in conjunction with ultrasonication for treating alfalfa sprouts enhanced the bactericidal effectiveness of EO water by 80%.

Fruits

Brown rot caused by Monilinia fructicola is one of the most destructive diseases of stone fruits (*32*). Treatment of non-wounded peach inoculated with

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Monilinia fructicola in EO water was reported to be capable of reducing incidence and severity of the disease (*33*). A treatment of peach in EO water for 5 min greatly reduced incidence and severity of the disease with no chlorine induced phytotoxicity formed on the treated fruits. Peach treated with EO water and held at 2°C with 50% relative humidity (RH) for 8 days did not develop brown rot until they were transferred to a storage condition of 20°C with 95% RH. Other studies of EO water as a disinfecting agent for reducing bacterial contamination on strawberry found that aerobic mesophiles on surfaces were reduced by 1.6 log CFU per strawberry after 10 min of EO water (32 ppm chlorine) treatment (*20*). The EO water treatment also reduced coliform bacteria and fungi on strawberry by >1.0 and 1.7 log CFU per strawberry, respectively. However, the treatment could not completely inactivate microorganisms from the surface of strawberry.

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Shell Eggs

The egg surface is easily contaminated with pathogens, such as *Salmonella* and *L. monocytogenes*, mainly from hen's feces and contaminated environments (*34*). Russell (*35*) investigated the bactericidal effects of EO water on reducing bacterial contamination on shell eggs using an electrostatic spray system and found that EO water could effectively eliminate pathogenic (*Salmonella* Typhimurium, *Staphylococcus aureus*, and *Listeria monocytogenes*) and indicator bacteria on the eggs. However, application of EO water as an egg disinfectant through the electrostatic spray system resulted in loss of 85-90% available chlorine and greatly reduced the efficacy of EO water on reducing bacteria (*36*).

Park *et al.* (*23*) investigated the efficacy of EO water treatment on reducing *S. enteritidis* and *L. monocytogenes* on shell eggs found that washing shell eggs inoculated with *S. enteritidis* (5.61 log CFU/egg) in EO water containing chlorine of 16, 41, and 77 ppm for 5 min resulted in reductions of the bacterium by 2.75, 3.50, and 3.48 log CFU/egg, respectively. Similar reductions (2.90, 3.47, and 4.00 log CFU/egg) were also observed for *L. monocytogenes* inoculated to eggs (7.03 log CFU/egg) after the EO water (16-77 ppm chlorine) treatment. A combined treatment of 1 minute ER water followed by 1 min EO water (77 ppm chlorine) yielded 3.66 and 4.39 log reductions of *Salmonella* and *Listeria* per shell egg, respectively. The reductions of *Salmonella* and *Listeria* per shell egg achieved by the combined wash with ER and EO water were found equivalent to reductions of *Salmonella* (3.81 log CFU per egg) and *Listeria* (4.01 log CFU per egg) observed after washing eggs with chlorinated water (200 ppm chlorine) for 1 min.

Poultry

Domestic fowl has been reported to be capable of harboring *L. monocytogenes* and serve as a potential vehicle for transmitting the pathogen to human (*37*). Poultry are frequently contaminated with human pathogen, such as *S. enteritidis* and *Campylobacter jejuni,* and raw poultry products have been reported to be responsible for many foodborne illnesses (*38*). Studies of *C. jejuni* contamination on chicken carcasses during slaughter showed that *C. jejuni* was detected on chicken skin and exposed carcass surfaces during slaughter operation (*39, 40*).

Rinsing poultry with chlorinated water, trisodium phosphate (TSP), or hot water are common interventions used by the meat and poultry industries to reduce bacterial contamination on carcass surfaces. These interventions have been found effective on reducing foodborne pathogens including *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* on meat or poultry surfaces (*41, 42, 43, 44, 45, 46, 47*). However, these interventions either require preparation of chemical solutions or have high operation costs.

Studies have been conducted to evaluate the efficacy of EO water on reducing *C. jejuni* and *Salmonella* contamination on poultry. Park *et al.* (*6*) investigated the effectiveness of EO water for inactivating *C. jejuni* on poultry. Results showed that washing chicken wings inoculated with *C. jejuni* (5.05 log CFU/g) with deionized water for 10 min at 23°C reduced the pathogen by 1.14 log CFU/g. However, washing chicken wings with EO water (50 ppm chlorine) was as effective as treatment of chlorinated water (50 ppm chlorine) on reducing *C. jejuni.* Populations of *C. jejuni* were reduced by 2.96 and 2.78 log CFU/g after the EO and chlorinated water treatments, respectively. No viable cells of *C. jejuni* were recovered from either EO or chlorinated water after the wash. Fabrizio *et al.* (*48*) investigated effects of EO water on reducing *Salmonella* Typhimurium on poultry and found that a treatment of EO water (pH:2.6, chlorine:20-50 ppm, ORP:1,150 mV) at 4°C for 45 min following refrigerated storage could reduce *S.* Typhimurium on poultry surfaces. Populations of *S.* Typhimurium on broiler carcasses (2.71 log CFU/ml of rinsate) were reduced by 0.83 log CFU/ml after the EO water treatment and to nearly undetectable levels after 7 d of storage at 4°C. Kim *et al.* (*22*) evaluated the efficacy of ER and EO water in preventing and removing fecal contaminants and killing *C. jejuni* on poultry carcasses under simulated industrial processing conditions. They found that ER water could be used to replace trisodium phosphates (TSP) in preventing attachment and removal of feces on surfaces of chicken carcasses. Immersion chicken carcasses in EO water significantly reduced the population of *C. jejuni* by 2.33 log CFU/g.

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Seafood Processing

Fresh seafood normally has a short shell life because of spoilage caused by growth of bacteria harbored on skin and gill. Keeping fresh fish in ice has been a common practice to preserve the quality of fish and retard the spoilage reaction. However, keeping fish in ice can not totally inhibit growth of bacteria, especial psychrotrophs that can grow at refrigeration temperatures. A post-harvest treatment that can reduce bacterial contamination on fish will increase the shell life of products.

EO water has been reported to be capable of reducing bacterial contamination on fish. Mahmoud *et al.* (*49*) studied the effects of EO water on reducing total bacteria count on fresh whole and skinless filleted carp and found that dipping whole carp in deionized water at 25°C for 15 min could only reduce total microbial count on skin by 0.6 log CFU/cm^2. However, the same treatment with EO water (pH:2.22, chlorine:40 ppm, ORP: 1,137 mV) resulted in 2.6 log CFU/cm^2 reduction of total microbial count. Similar results were observed when filleted carp was studied. The EO water treatment reduced total microbial count on the fillet by 1.9 log CFU/cm^2 while only 0.3 log CFU/cm^2 reduction was achieved by the deionized water treatment. These results indicate that EO water can be used to reduce bacterial populations on fish and extend shell life of products. Huang *et al.* (*50*) studied EO water for reducing microbial population on tilapia and platform of fish retailer. They reported that EO water achieved additional 0.7 log CFU/cm^2 reduction of *E. coli* than tap water after 1 min of immersion treatment. The EO water treatments also reduced *Vibrio parahaemolyticus* on tilapia by 1.5 and 2.6 log CFU/cm^2 after 5 and 10 min, respectively. In addition, treatments of EO water also effectively disinfected the platform of fish retailer in traditional and fish markets.

Surface Sanitizer

Food-processing counters and kitchen cutting boards are potential sources of cross-contamination during food preparation (*51*). Several studies have reported that EO water could be used as a sanitizer to reduce bacterial contamination on food processing and contact surfaces. Studies conducted by Venkitanarayanan *et al.* (*24*) found that soaking plastic cutting boards inoculated with *E. coli* O157:H7 in EO water (pH:2.5, chlorine: 80 ppm, ORP: 1,165 mV) at 23°C for 10 min reduced bacterial population by about 5.1 log CFU/100 cm^2, while only about 0.9 log CFU/100 cm^2 reduction was achieved by soaking the boards in deionized water. The populations of *E. coli* O157:H7 on cutting boards were completely inactivated with soaking in EO water at 35°C for 20 min or 45°C for 10 min. The study also found that populations of *L. monocytogenes* inoculated to cutting boards were reduced by 4.8 CFU/100 cm^2 after an EO water treatment at 23°C for 20 min. Both *E. coli* O157:H7 and *L.*

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monocytogenes were recovered from the deionized water used for soaking cutting boards, but were not detected in EO water after treatments. These results suggest that soaking cutting boards in EO water could be an effective means to inactivate foodborne pathogens and prevent cross-contamination during food preparation.

The effects of EO water on reducing bacterial contamination on surfaces of glass, stainless steel, glazed and unglazed ceramic tiles, and vitreous china have also been studied. Immersion treatments of those materials inoculated with *Enterobacter aerogenes* and *Staphylococcus aureus* in EO water for 5 min without agitation resulted in 2.2 to 2.4 log CFU/cm^2 reductions of *E. aerogenes* and 1.7 to 1.9 log CFU/ cm^2 reductions of *S. aureus*, whereas treatment with water only resulted in 0.1 to 0.3 log CFU/ cm^2 reduction for both bacteria (*25*). In addition, EO water was reported effective in eliminating *L. monocytogenes* biofilms on stainless steel (*52*). A 5-min EO water treatment reduced the adherent cell population on the stainless steel surface by about 9 log cycles. Ayebah *et al.* (*26*) also reported that treatments of EO water for 30-120 s resulted in 4.3 to 5.2 log CFU/10 cm^2 reductions of viable *L. monocytogenes* cells in biofilms on stainless steel surfaces. The investigators also reported that a combined treatment of ER water followed by EO water produced an additional 0.3 to 1.2 log CFU/10 cm^2 reduction of *L. monocytogenes* in biofilms than by EO water treatment alone.

A similar study was conducted by Liu *et al.* (*14*) to investigate the antibacterial activity of EO water against *L. monocytogenes* on clean and soil-containing surfaces. *L. monocytogenes* was inoculated to surfaces of stainless steel, ceramic tile, and floor tile cut into small chips (5×5 cm^2) and soaked in EO water (pH:2.5, chlorine:50 ppm, ORP:1,150 mV) at room temperature for 5 min. Results showed that EO water was effective in reducing *L. monocytogenes* on clean surfaces, but was less effective when food residue was attached to surfaces. Treatments of clean surfaces with EO water resulted in 3.73, 4.24, and 5.12 log CFU/chip reductions of *L. monocytogenes* on stainless steel, ceramic tile, and floor tile, respectively. However, only moderate reductions of 2.33, 2.33, and 1.52 log CFU/chip on stainless steel, ceramic tile, and floor tile, respectively, were achieved by the EO water treatment when crabmeat residue was attached to surfaces. These results indicate that EO water could be used as a sanitizer to reduce *L. monocytogenes* contamination on food-processing surfaces. However, the efficacy of EO water as a sanitizer can be reduced by presence of organic materials.

To ensure the efficacy of EO water on inactivating bacteria, food ingredients attached to surfaces of equipments or processing counters need be removed by thorough cleaning before the EO water treatment. Both ER and EO water can be used as cleaning aids to remove certain food ingredients from surfaces. ER water was reported to be more effective than warm water for removing gelatin on equipment surfaces with an optimal temperature for

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cleaning at 50°C, while EO water could be used to remove calcium hydrogenphosphate deposited on stainless steel particles (*53*).

Summary

Many disinfectants are commercially available for disinfecting food-processing surfaces. Among them, chlorine is the most widely used sanitizer because of its broad-spectrum bactericidal activities and relatively low cost (*54*). However, a major disadvantage of using chlorine-based compounds as sanitizers is that workers must prepare a diluted working solution by handling concentrated chemicals, which is a safety concern.

EO water has been demonstrated to be an effective disinfecting agent and can be used as a chlorine alternative for reducing microbial contamination on raw materials and processing surfaces. Major advantages of using EO water as an anti-microbial agent include: (1) the EO water treatment is as effective as the chlorinated water treatment, (2) there is no need for handling potentially dangerous concentrated chemicals, (3) the apparatus is easy to operate and relatively inexpensive, (4) the process is environmentally friendly because production of EO water uses only water and sodium chloride, and (5) the properties of EO water can be controlled at the site of production.

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Chapter 15

Use of Mannitol Hydrogen Peroxide to Control Microbial Produced Skin Irritants

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Production of irritants by microbes on or near the skin's surface may lead to skin inflammation as well as offensive odors. A mannitol hydrogen peroxide (MHP) mixture was found to reduce the production of skin irritating compounds by facultative Gram-negative bacteria. This control method appears to influence metabolic regulation rather than impacting microbial viability, an aspect important to the overall maintenance of normal skin microflora.

A mixture of hydrogen peroxide and mannitol was heated for 7 hr at 97 °C resulting in a white granular solid. A 450-fold decrease in specific ammonia production was observed when *Proteus mirabilis* was grown in the presence of urea and MHP as compared to cells in urea alone. Furthermore, it was observed that MHP produced an 8- and 2- fold decrease in release of cytokines IL-1α and IL-8, respectively, in a *P. mirabilis* challenged skin model. It is hypothesized that MHP provides a sustainable discharge of oxygen that shifts the metabolism of the bacterium. This bacterial control system can be applied to personal and health care products to control odors and reduce bacterial induced skin irritation, all without employing harmful antimicrobials.

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Introduction

Antimicrobials have been incorporated into a wide range of consumer products over the years (*1, 2*). Since most antimicrobials are toxins by definition, they can have a negative impact on skin health, causing allergic reactions, stinging, discoloration, as well as drying of the skin (*3, 4*). In addition, it has been hypothesized that the widespread use of antimicrobials can provide selective pressures that favor the emergence of bacterial strains resistant to antimicrobials, including clinically relevant antibiotics (*2, 5, 6*). Taken together, a need exists to develop non-toxic approaches to microbial control. The ultimate topical antimicrobial would be one that eliminates the problem microbe and associated skin irritants, is gentle to the skin, and does not promote the development of antimicrobial resistance. To this end, we have developed a non-toxic mixture that lessens the production of skin irritating compounds by *Proteus mirabilis*, a Gram-negative bacterium that is implicated with many health related problems (*7*).

Skin inflammation is the result of immunological events occurring in the viable layers of the skin. Inflammation is initiated by the production and release of pro-inflammatory mediators that result in the recruitment and activation of circulating leukocytes (*8*). This process eventuates in the hallmark features of skin inflammation such as redness, swelling, and adverse polysensorial events. It is generally accepted that numerous molecules and microbial processes can elicit skin inflammation (*9*).

While consumers are looking for products that will decrease the risk of infection, they also appreciate that other benefits are attendant with antimicrobials such as odor control. What is not well appreciated is that microbes can produce metabolites that will lead to inflammation of the skin; some of these are volatile compounds.

Recently, *in vitro* studies have demonstrated that vapor phase compounds can elicit the production of pro-inflammatory factors in skin models (*10*). Some of these molecules, such as oxalacetic acid, isovaleric acid, butyric acid, propionic acid, and hexanoic acid, are associated with the anaerobic growth of bacteria (*11*). While many of the odor molecules produced by microorganisms are organic in nature, there are inorganic compounds such as ammonia and hydrogen sulfide that are also generated by bacterial growth (*12, 13, 14, 15*).

Non-toxic technologies that reduce microbial production of irritants at or near the skin surface will have significant consumer benefits. The inclusion of these technologies in personal care products can reduce skin inflammation and objectionable odors. A number of bacterial virulence factors are associated with the anaerobic state of the cell (*16, 17*). By providing the appropriate aeration during growth, it may be possible to control the negative attributes of these pathogens. This chapter describes the use of mannitol hydrogen peroxide to reduce the amount of unwanted metabolites produced by *P. mirabilis* by

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influencing metabolic regulation rather than viability, an aspect that can be important to the overall maintenance of normal skin microflora.

Materials and Methods

Mannitol Hydrogen Peroxide

Hydrogen peroxide (22.5 ml of 30% hydrogen peroxide, Sigma Chemical, St. Louis, MO) was mixed with mannitol, hexane-1,2,3,4,5,6-hexol, (15 g, Sigma Chemical) in a 300 ml Pyrex Fleaker (Corning, Acton, MA). Mannitol was dissolved completely in the hydrogen peroxide after which the uncovered Fleaker containing the solution was placed in a 97 °C forced air oven for 3 to 24 hr. The solution evaporated resulting in a white granular solid. Multiple batches were prepared with residence times in the oven of 3, 4.5, 7, and 24 hr. Unless noted otherwise, experiments reported here employed a mannitol hydrogen peroxide (MHP) complex having an oven residence time of 7 hr.

Bacteria and Analytical Measurements

Proteus mirabilis (ATCC 29906, American Type Culture Collection, Manassas, VA) was recovered from a frozen state by loop transfer to 10 ml of trypticase soy broth (TSB, Difco, Ann Arbor, MI) in a 15 ml loosely tightened screw capped conical polystyrene tube (VWR, West Chester, PA) and grown stationary overnight at 37 °C. The subsequent suspension was streaked for isolation on the surface of trypticase soy agar (TSA, Difco) and incubated overnight at 37 °C. From this plate a single colony was transferred to 10 ml of TSB in a 15 ml sterile screw capped conical tube and incubated 18 hr at 37 °C under facultative conditions, a stationary tube with a loose cap. The resulting cell suspension was used to initiate experiments. For most experiments a 9:1 mixture of 0.22 µm filter (Gelman, Ann Arbor, MI) sterilized urine to TSB was used to grow *P. mirabilis*. All urine used in these studies was a pooled mixture obtained from a minimum of 4 adult human females.

After the experimental growth period, post incubation culture broth was analyzed to determine: (a) bacterial yields, (b) the amount of ammonia in the growth solution, (c) pH, and (d) induction of interleukin 1, alpha (IL-1α) and interleukin 8 (IL-8) in the EpiOcular™ skin model (MatTek Corporation, Ashland, MA). Bacterial yields were determined by measuring optical density (OD) of the culture broth at 660 nm with a 0.2 cm light path (SpectraMax M5, Molecular Devices, Sunnyvale, CA) and relating OD to colony forming units (CFU) to calculate cell densities (*18*). Ammonia in the solution was measured using an ammonia combination probe (Beckman, Fullerton, CA) and an Orion

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pH meter (Orion, Boston, MA) calibrated with ammonia standards (Orion). Post incubation culture broth used to insult the ski model was filter-sterilized with a 0.22 µm filter (Gelman) before use. All pH measurements were done using an Orion pH meter (Orion).

EpiOcular™ Skin Model and Measurement of Irritation Response

The EpiOcular™ skin model (OCL-200, MatTek Corporation) was used to determine the ability of post incubation culture broth to induce pro-inflammatory events. This skin model consists of normal, human-derived epidermal keratinocytes, which have been cultured to form a stratified, squamous epithelium (*19*). The epidermal cells were incubated on semi-permeable membranes within cell culture inserts using serum free Dulbecco's Modified Eagle's medium until the day of the insult experiment. On the day of the insult experiment, one culture insert was placed in each well of a 6 well cell culture plate (Falcon, BD Biosciences, San Jose, CA), containing 1 ml of the pre-warmed media. The plates were covered and placed in a 37 °C, 5% CO_2 atmosphere for 30 min. After this pre-incubation, the skin model was insulted with 25 µl of the filter sterilized post incubation culture broth or other test sample for 6 hr at 37 °C, in a 5% CO_2 atmosphere. Following the insult, skin model culture medium was removed and placed in a sterile 1.2 ml polypropylene freezer vial (VWR), then immediately placed in a -70 °C freezer until processing for measurement of pro-inflammatory cytokines.

The cytokines IL-1α and IL-8 were quantified using the appropriate Quantikine® enzyme linked immunosorbant assay (ELISA) kit (R&D Systems, Minneapolis, MN). Viability of the skin model was evaluated using metabolic activity as an endpoint (*19*). Briefly, the skin model was incubated after the insult period with a skin culture medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical) and subsequently used to relate metabolic reduction of MTT to viability.

Effect of Mannitol Hydrogen Peroxide on Growth of *P. mirabilis*

P. mirabilis cultured overnight in TSB was transferred to a urine-TSB mixture (9:1) and incubated under facultative conditions for 18 hr, as described previously. A 10% MHP solution (w/v) of each drying time was prepared by mixing 1 g of MHP with 10 ml urine-TSB (9:1) mixture in a 15 ml screw top conical tube. This mixture was used to make a series of exposure concentrations in a 96 well plate (Falcon). Each well contained 200 µl of growth media to which 1 µl of enriched *P. mirabilis* culture in the urine-TSB suspension (10^9 CFU/ml) was added, except for the negative control wells to which 1 µl of

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urine-TSB was added. The mixtures were incubated stationary for 48 hr at 37 °C. After incubation, post incubation culture broth was analyzed for bacterial cell growth by measuring the OD of the suspensions at 660 nm employing a 0.2 cm light path (SpectraMax M5, Molecular Devices).

Impact of Aeration on Ammonia and Skin Irritant Production by *P. mirabilis*

P. mirabilis was grown under various oxygen conditions to determine the impact on bacterial metabolism. For bacteria grown under aerobic conditions, 50 µl of *P. mirabilis* was taken from an 18 hr urine-TSB culture, prepared as previously described, and added into 50 ml of urine-TSB in a cotton-stoppered 250 ml flask (Corning). This mixture was incubated with shaking at 250 rpm at 37 °C for 18 hr. For bacteria grown under facultative conditions, 10 µl of *P. mirabilis* was taken from 18 hr urine-TSB culture and inoculated into a 10 ml volume of urine-TSB in a 15 ml tube conical tube, loosely fitted with a screw cap, and held stationary at 37 °C for 18 hr. For bacteria grown under anaerobic conditions, 10 µl of *P. mirabilis* was taken from 18 hr urine-TSB culture and inoculated into a 10 ml volume of urine-TSB (9:1) in a 15 ml conical tube. The tube was purged with nitrogen and a screw cap tightly closed and incubated overnight at 37 °C for 18 hr. Upon completion of the incubation period, samples were assayed for cell yields, ammonia, and the ability of filter-sterilized post incubation culture broth to elicit an IL-1α release by the skin model.

Ability of Mannitol Hydrogen Peroxide to Alter Urea Hydrolysis and Skin Irritant Production by *P. mirabilis*

P. mirabilis was exposed to three concentrations of MHP (0%, 5%, and 10% w/v) for 18 hr in the urine-TSB broth at 37 °C with facultative conditions, as described previously. Upon completion of the incubation period, samples were assayed for cell yields, ammonia, and the ability of the filter sterilized post incubation culture broth to elicit IL-1α and IL-8 release by the skin model.

Urease Hydrolysis of Urine Mixture

The urine-TSB was incubated with a urease to release ammonia independent of cell growth. A 50 ml sterile screw capped conical tube (VWR) containing 35 ml of urine-TSB was mixed with 0.1 g of *Canavalia ensiformis* urease (Sigma Chemical, Type III, 15000-50000 units/g) and incubated for 18 hr at 37 °C. After incubation the solution was analyzed for: (a) amount of

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ammonia in solution, (b) pH, and (c) induction of IL-1α and IL-8 in the skin model.

Results

To find the mannitol hydrogen peroxide (MHP) mixture that was not lethal to *P. mirabilis*, various concentrations of MHP and mannitol were evaluated in the presence of facultatively cultivated *P. mirabilis*. The MHP utilized for these experiments was subjected to different drying times from 3 to 24 hr. MHP at and below 10% (w/v) dried for 3 hr at 97 °C had a significant impact on *P. mirabilis* growth (Table I). In contrast, MHP subjected to heating for at least 7 hr at 97 °C appeared to have little effect on the growth of the bacteria.

Table I. Mannitol Hydrogen Peroxide Dry Time Impact on Growth

Drying Time (hr)	*Cell Yield (OD 660 nm)*					
	% (w/v) Mannitol Hydrogen Peroxide					
	10	*5*	*2.5*	*1.25*	*0.625*	*0*
24	0.80	0.88	0.93	0.95	0.96	1.00
7	0.82	0.90	0.97	0.98	0.99	0.98
4.5	0.04	0.87	0.97	0.99	0.93	1.00
3	0.03	0.03	0.03	0.04	0.99	0.98

Mannitol mixed with hydrogen peroxide was dried at 97 °C at the times listed. *P. mirabilis* grown in urine-TSB with concentrations of MHP listed at 37 °C for 48 hr. Units are optical density (OD 660 nm).

P. mirabilis was grown under various conditions to determine whether aeration affected the amount of ammonia production per cell and subsequent release of IL-1α in the skin model by post incubation culture broth. Ammonia production was found to be inversely proportional to the available oxygen during growth (Table II). Furthermore, increasing aeration during growth appeared to substantially reduce IL-1α released by the skin model. In contrast, aeration had no detectable effect on *P. mirabilis* cell yields (Table II). Culture broth without bacteria did not illicit a detectable IL-1α release in the skin model (data not shown). Likewise, viability of skin model, as measured by MTT reduction, was not impacted following exposure to post incubation culture broth (data not shown).

The addition of MHP decreased the amount of ammonia production per cell with *P. mirabilis*. Furthermore, this interaction appears to be dosing dependent, shown by decreasing ammonia in solution with increasing levels of MHP exposure (Table III). Likewise, post incubation culture broth from *P. mirabilis*

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Table II. Aeration Impact on Growth, Ammonia, and Cytokine Release

Growth Condition	*Bacterial Yield (CFU/ml)*[a]	*Ammonia/Cell (ppm/CFU x 10^8)*	*EpiOcular™ IL-1α (pg/ml)*[a]
Aerobic	$3.9 \times 10^9 \pm 8 \times 10^7$ Change data to $\log_{10}$ value such as 9.59 ± SD	2.3	26 ± 4
Facultative	$1.3 \times 10^9 \pm 8 \times 10^7$ Change to log value	4.5	164 ± 49
Anaerobic	$1.1 \times 10^9 \pm 3 \times 10^7$ Change to log value	7.0	161 ± 47

P. mirabilis was grown in urine-TSB at 37 °C for 18 hr. Ammonia was measured in culture media after termination of growth. Release of IL-1α from skin model was measured after exposure to filtered sterilized post incubation culture broth.

[a] Mean of triplicate values ± standard deviation.

Table III. Inhibition of Ammonia Production and Cytokine Release

MHP[a] *(% w/v)*	*Ammonia/Cell (ppm/CFU x 10^8)*	*EpiOcular™ IL-1α (pg/ml)*[b]	*EpiOcular™ IL-8 (ng/ml)*[b]
0	4.5	125 ± 19	27 ± 2
5	1.6	48 ± 9	16 ± 8
10	0.01	15 ± 13	9 ± 2

P. mirabilis grown in urine-TSB at 37 °C for 18 hr with mannitol hydrogen peroxide. Ammonia was measured in culture media after termination of growth. Release of IL-1α and IL-8 from skin model was measured after exposure to filtered sterilized post incubation culture broth.

[a] MHP = Mannitol hydrogen peroxide.

[b] Mean of triplicate values ± standard deviation.

exposed to MHP decreased IL-1α and IL-8 release in the skin model. In contrast, EpiOcular™ challenged with urea-TSB reacted with *C. ensiformis* urease did not induce pro-inflammatory response in the skin model (data not shown).

P. mirabilis grown in urine-TSB increased the pH in all evaluated aeration conditions (Table IV). A similar response was observed for the mixture of urine-TSB and *C. ensiformis* urease. The addition of MHP to urine-TSB appears to lower the pH from 6.8 to around 5. Growth of *P. mirabilis* increased the pH of the mixture, while growth in 10% (w/v) MHP did not increase the pH as much as was observed in 5% (w/v) MHP (Table IV).

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Table IV. Impact of Growth of *P. mirabilis* on pH

Growth Condition	*Before Growth*	*After Growth*		
		Aerobic	*Facultative*	*Anaerobic*
Urine-TSB	6.8	9.0	9.1	9.1
Urine-TSB + 5% MHP[a]	5.2	ND	9.2	9.1
Urine-TSB + 10% MHP	4.9	ND	5.8	8.7
Urine-TSB + Urease	6.8		9.2[b]	

[a] MHP = Mannitol hydrogen peroxide
[b] No bacteria present
ND = Not determined
Units are pH

Discussion

P. mirabilis grown in the presence of mannitol hydrogen peroxide (MHP) decreased the ability of the post incubation culture broth to irritate skin (Table III). As antimicrobial action of MHP was not detected, the reduction in skin irritant production is likely related to a change in bacterial metabolism rather than growth inhibition. It is hypothesized that MHP reduces microbial produced skin irritants by generating oxygen, shifting bacterial metabolism to an aerobic respiratory pathway. Therefore, it is believed that the control function is related to physiological events associated with aerobic rather than anaerobic respiration (*16, 20, 21*). Indeed, a number of virulence factors for *P. mirabilis* and other bacteria are associated with the aerobic/anaerobic state of the cell (*17, 22*). Furthermore, anaerobic to aerobic metabolism shift directly alters physiological regulatory functions by altering the overall energy balance of the cell (*23*). While the precise mechanism by which MHP acts is unknown, the observations in this study suggest that aerobic growth reduces the production of skin irritants by *P. mirabilis*.

The conditions used to make MHP are critical to provide a composition that alters cell metabolism without imparting antimicrobial effects (Table I). Combinations of polyols and hydrogen peroxide can provide oxidative species capable of killing bacteria (*24*). However, the MHP produced by drying the mannitol and hydrogen peroxide at 97 °C for >7 hr did not exhibit detectable antimicrobial attributes (Table I). In contrast, drying under these conditions for ≤ 4.5 hr resulted in antimicrobial compositions.

It is thought that the extended drying times have the effect of reducing the amount of reactive radicals released from the MHP during use allowing for mannitol to serve as a radical scavenging reservoir (*6, 25*). In contrast, reduced MHP drying time produces excess radicals that overcome this reservoir leading to a composition lethal to bacteria. Appropriately dried MHP is oxygenic, shifting microbial metabolism to aerobic respiration.

It is further hypothesized that when the MHP is exposed to moisture during use, it decomposes and gradually releases oxygen, water, and possibly small amounts of peroxide and oxygen radicals. During decomposition, the mannitol acts as a reducing agent scavenging any free radicals present in the mixture. The oxygen produced by MHP decomposition and the attendant shift in bacterial metabolism decreases the production of the skin irritants (Tables II and III).

A MHP mediated shift in bacterial metabolism from anaerobic to aerobic appears to have multiple impacts on bacterial physiology, altering ammonia production from urea and reducing the discharge of pro-inflammatory factors in skin exposed to post incubated culture broth (Table II). It is known that production of urease in bacteria can be up-regulated by either acidic conditions or nitrogen starvation (*13*). The conditions used in these studies are most likely not nitrogen limited due to the complexity of the TSB. Interestingly, the data indicates that pH increases even as ammonia production is reduced (Table III and IV) implying that other regulatory mechanisms account for these observations.

Addition of oxygen to the growth environment through increasing agitation (Table II) has a similar effect on *P. mirabilis* ammonia production as was observed with *P. mirabilis* grown in the presence of MHP (Table III). These observations are consistent with the hypothesis MHP is oxygenic, shifting bacterial metabolism to aerobic respiration resulting in a possible contemporaneous reduction in urease production. Further studies are planned to determine the impact of dissolved oxygen levels in the media during growth to explore this hypothesis. Likewise, monitoring pH during growth may ascertain if pH is a controlling factor or that the pH values obtained here are artifacts of stationary growth combined with low buffering capacity of the media.

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Skin irritation caused by *P. mirabilis* appears to be independent of hydrolysis of urea by urease as topical application of urine-TSB hydrolyzed by *C. ensiformis* urease to the skin model did not induce pro-inflammatory events (data not shown). Conversely, topical application of post incubated culture broth generated by increasing aeration (Table II) or inclusion of MHP (Table III) with *P. mirabilis* cultures resulted in reduced release of pro-inflammatory cytokines in the skin model relative to the respective controls. Taken together, MHP appears to have multiple positive effects for controlling unwanted bacterial by-products.

P. mirabilis is known to have a swarming cell type that is implicated in pathogenesis (*17, 26*). This type of behavior is closely associated with anaerobic growth and a shift to aerobic growth for these cells will limit swarming behavior (*17, 20, 26*). As these effects appear to be closely associated with oxygen modulated ribonucleotide reductase regulation, it is possible that MHP directly influences the shift to aerobic ribonucleotide reductase allowing for repression of putative virulence factors that elicit skin inflammation (*20, 22*).

Bacteria that produce irritants are present on the skin, mucus membranes, and in certain bodily waste products such as feces (*9*). Moreover, other bodily wastes and various exudates, such as urine and sweat, respectively, provide carbon and nitrogen sources for microorganisms (*9*). The presence of these materials can enhance facultative and anaerobic bacterial growth depending on the local micro-environment. As a result, when insufficient amounts of oxygen are present to serve as a terminal electron acceptor, facultative anaerobic bacteria present on and near the skin can produce volatile and non-volatile compounds that can elicit skin irritation. This situation can occur in a variety of personal and health care situations relevant to, for example, diaper, vaginal, wound, underarm, and oral care products.

Traditional methods of microbial control are typically non-selective, in that bacteria are killed indiscriminately, whether the bacteria are beneficial or not (*1, 2*). In the oral environment, chronic periodontitis may be the result of a global perturbation of the oral bacterial ecology (*27*). Likewise, vaginal health is tightly linked to the balance of an appropriately balanced micro-ecology (*28, 29*), an ecology that can be profoundly altered by the application of nonselective pressures due to microbial control agents. It is possible that in both these cases MHP can be used to alter the metabolism of the negative or problem micro-flora such that reduction of anaerobically produced virulence factors would limit production of irritants and possibly pathology. In conclusion, the use of MHP in personal and health care products could provide for a means to control bacterial irritants and odors without adversely altering the micro-ecology of the skin or mucous membranes.

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Chapter 16

Micrology and Chemistry of *ortho*-Phthalaldehyde

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Introduction

The use of *ortho*-phthalaldehyde (OPA) as an active ingredient in a high-level disinfectant is a relatively recent occurrence and as such there is little information on the antimicrobial activity. This paper is intended to summarize the information that is currently available

The antimicrobial properties of OPA have been described (1) and its spectrum of activity and mechanism of action studied (2,3,4,5,6). OPA has been shown to be effective against a wide variety of microorganisms, including vegetative bacteria, mycobacteria, fungi, and viruses. OPA when formulated at 0.5% also demonstrates activity against bacterial spores but with extended exposure times or higher temperatures (6,7). With the emergence of glutaraldehyde-resistant mycobacteria (8), the importance of new antimicrobial active ingredients cannot be underscored.

Chemistry

Structure

Glutaraldehyde, 1,5-pentanedial, is an aliphatic dialdehyde (**A** in Figure 1). It was introduced commercially as a high-level disinfectant and sterilant during the 1960's and is still widely used. Several other aliphatic dialdehydes display biocidal activity, but they lack activity against bacterial spores. Succinaldehyde, 1,4-butanedial (**B** in Figure 1), has found limited commercial application as a disinfectant.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

OPA, 1,2-benzenedicarboxaldehyde, is an aromatic dialdehyde (**C** in Figure 1). OPA was introduced only relatively recently as the active ingredient in a high level disinfectant (1). The two isomers of OPA, *meta*-phthalaldehyde and *para*-phthalaldehyde (**D** and **E**, respectively, in Figure 1) lack significant biocidal activity by themselves, although they have been found to enhance the mycobactericidal activity when added to glutaraldehyde (9).

In aqueous solutions of glutaraldehyde, the free monomeric dialdehyde is in equilibrium with a mixture of hydrated species, predominantly the cyclic hemiacetal in equal amounts of *cis* and *trans* stereoisomers (**F** in Figure 2) (10). Other species in the equilibrium mixture include the linear monohydrate, linear dihydrate, and polymers of the cyclic hemiacetal (11).

OPA in aqueous solution exists predominately as cyclic monohydrate (**G** in Figure 3) in equilibrium with the free monomeric dialdehyde (12).

Mechanism of action

The mechanism of action of OPA is based on the chemistry of dialdehydes, but is strongly influenced by the aromatic ring to which the aldehyde groups are attached. The antimicrobial activity of both OPA and glutaraldehyde is a result of the aldehyde groups reacting with amine groups on proteins, nucleic acids and other microbial structures such as cells walls. Some differences between the chemical reactions of glutaraldehyde and OPA that might account for the differences in microbicidal activity are (a) that glutaraldehyde will not react with histidine whereas OPA will, presumably due to Van der Waals interaction between the benzene ring on OPA and the imidazole ring on histidine; (b) aromatic aldehydes (OPA) are less reactive than aliphatic aldehydes (glutaraldehyde) in nucleophilic addition reactions; (c) OPA has a rigid planar aromatic structure whereas glutaraldehyde is flexible thereby having the ability to orient itself to react with peptide chains; and (d) OPA can only react with primary amines whereas glutaraldehyde can react with primary and secondary amines (3). The lipophilic nature of OPA provided by the aromatic ring is thought to provide additional antimicrobial activity because it allows for better penetration through the outer cell surface structures of mycobacteria and gram-negative bacteria.

Stability

Most glutaraldehyde-based high-level disinfectants are formulated as a two-component product, consisting of acidic solution of 2.4 – 3.4% glutaraldehyde to which an alkaline buffering agent is added, resulting in a slightly alkaline solution (pH 7.5 to 9) that has maximum sporicidal activity. However, Aldol condensation polymerization of glutaraldehyde occurs slowly at this alkaline pH

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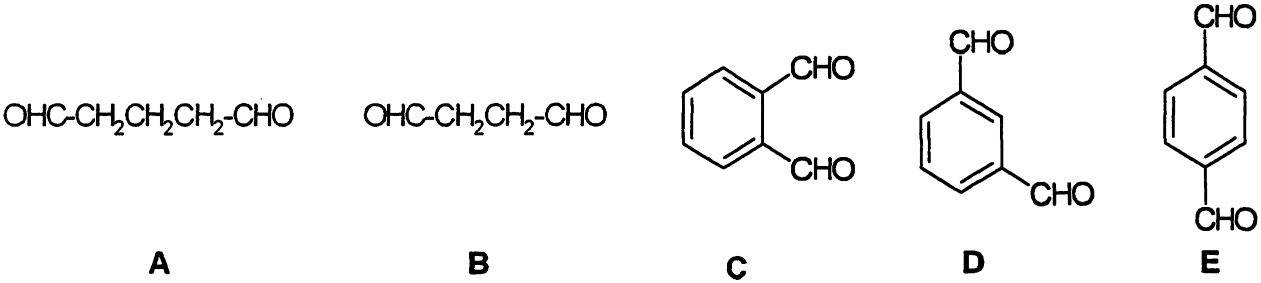
OHC-CH2CH2CH2-CHO
OHC-CH2CH2-CHO
CHO
CHO
CHO
CHO
CHO
CHO
A
B
C
D
E

Figure 1. Structure of aldehydes (A) glutaraldehyde, (B) succinaldehyde, (C) ortho-phthalaldehyde, (D) meta-phthalaldehyde, (E) para-phthalaldehyde

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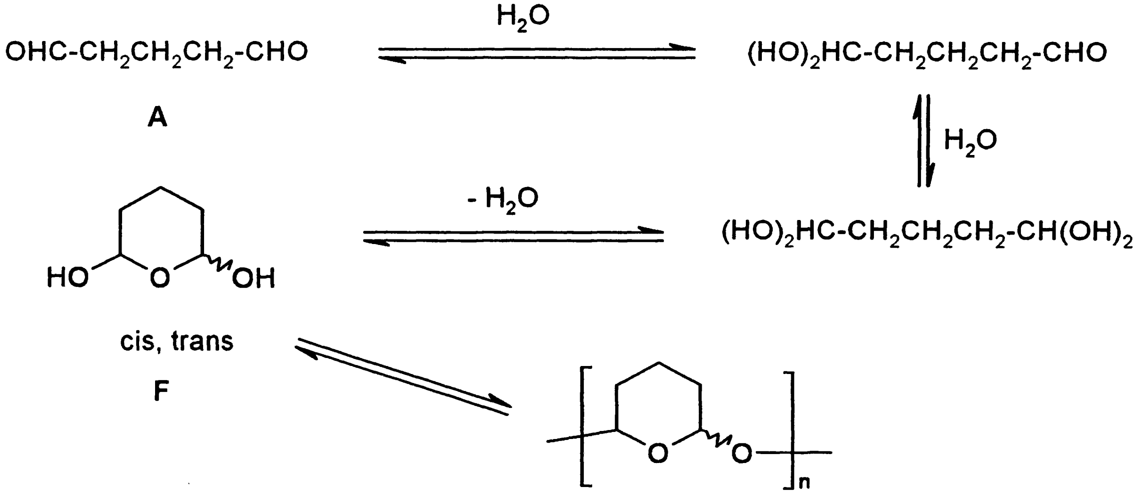
OHC-CH2CH2CH2-CHO
A
H2O
(HO)2HC-CH2CH2CH2-CHO
H2O
- H2O
(HO)2HC-CH2CH2CH2-CH(OH)2
HO
O
OH
cis, trans
F
O
O
n

Figure 2. Chemical structure of various species of glutaraldehyde in aqueous solutions (A) free monomeric glutaraldehyde, (F) cis- and trans- stereoisomers of cyclic hemiacetal

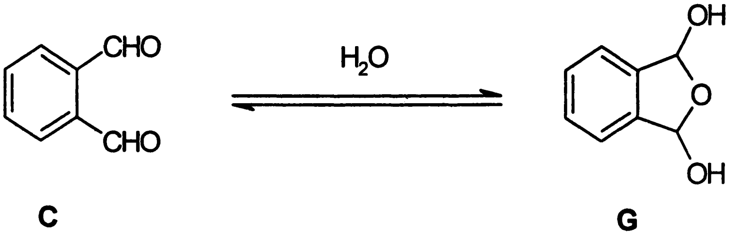

Figure 3. Chemical structure of various species of OPA in aqueous solutions (C) monomeric ortho-phthalaldehyde, (G) cyclic monohydrate

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(H in Figure 4). Because of this chemical degradation, alkaline glutaraldehyde solutions are labeled with a maximum "use life," typically 14, 28, or 30 days.

Since the OPA molecule lacks an alpha-hydrogen, the Aldol condensation is not possible. Therefore, in aqueous solution OPA is stable over a wider pH range, from acidic (pH 3) to alkaline (pH 9). Commercially available high level disinfectant product with OPA as the active ingredient is formulated as one component containing 0.55% OPA at near neutral pH (7.5). Since OPA does not degrade chemically during its use life, it can be formulated at a lower initial concentration, and dilution becomes the main factor in determining its use life. OPA can undergo an intramolecular Cannizzaro reaction, but this reduction-oxidation process only occurs at a very alkaline pH (> 10) and elevated temperatures (13). Since commercially available solutions are formulated at much lower pH and used at room temperature, this reaction is not likely to interfere with the chemical stability of products.

Disposal

Glutaraldehyde solutions can be chemically inactivated by addition of ammonia or ammonium salts, by increasing the pH to 11 – 12 to force Aldol polymerization, or by addition of sodium bisulfite, $NaHSO_3$ to form the bisulfite adduct (14). Spent OPA solution can be chemically inactivated with glycine, free base (15).

Microbiology

Sporicidal Activity

A comparison of the sporicidal activity of glutaraldehyde with OPA at the concentrations at which they are commercially formulated indicates that glutaraldehyde is considerably more sporicidal. It should be noted that the

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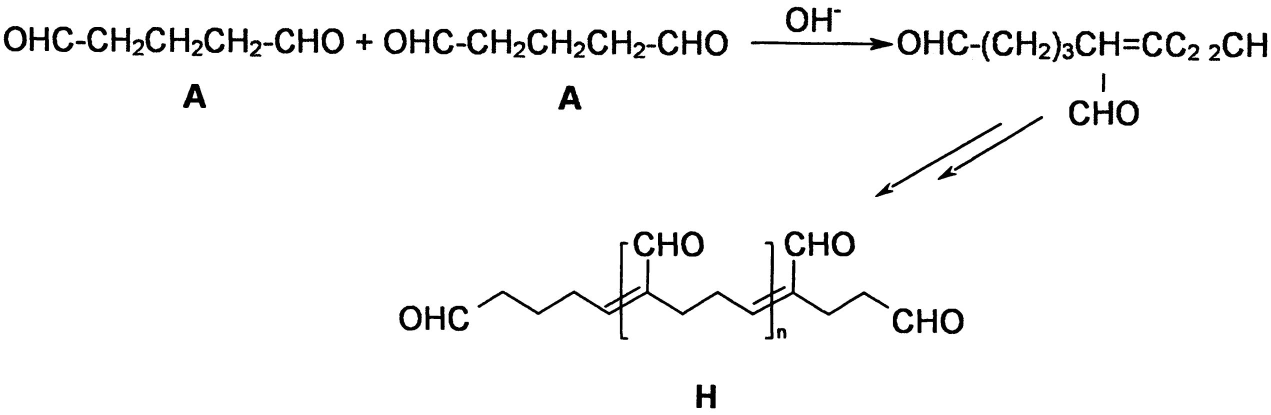
OHC-CH2CH2CH2-CHO + OHC-CH2CH2CH2-CHO
A
A
OH-
OHC-(CH2)3CH=CC2 2CH
CHO
CHO
CHO
OHC
CHO
n
H

Figure 4. Aldol condensation polymerization (H) of glutaraldehyde (A).

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difference in sporicidal activity between 2% glutaraldehyde solutions and 0.55% OPA solutions may be strictly a result of the number of reactive aldehyde group. Two and three percent glutaraldehyde solutions achieved a 6 log10 reduction in spores of ***Bacillus subtilis*** in 6 and 3 hours respectively while 0.3 and 0.55% OPA required 72 and 48 hours respectively. The activity of OPA against ***B. subtilis*** spores in a suspension test is directly related to exposure temperature as shown in Figure 5. At 30°C 0.3% OPA demonstrated a 5 $\log_{10}$ reduction in 8 hours whereas at 35°C a 6 $\log_{10}$ reduction is achieved in 3 hours. Walsh, Maillard and Russell (2) demonstrated a direct relationship with pH and concentration and sporicidal activity with ***B. subtilis*** spores in both suspension and carrier tests. A 2% solution of OPA at pH 8, 25°C showed a >5 $\log_{10}$ and >6 $\log_{10}$ in 4.5 hours, in suspension and carrier tests, respectively.

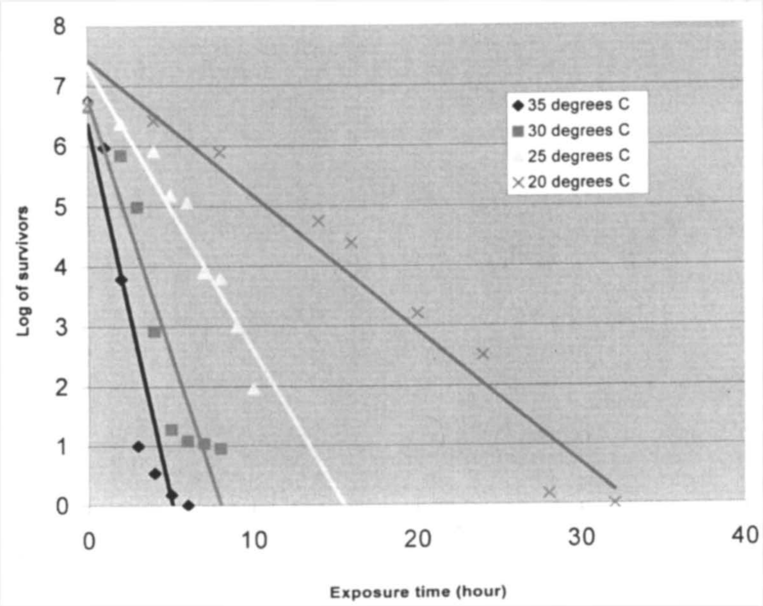

Figure 5. Regression results of quantitative suspension test with Bacillus subtilis *in 0.3%* ortho-*phthalaldehyde at various temperatures*

With spores of *Clostridium tetani*, 2 and 3% glutaraldehyde achieved a 2 $\log_{10}$ reduction in 2 min while 0.3 and 0.55% OPA achieved the same results in 20 and 5 min respectively. Finally with spores of *C. difficile*, 2 and 3% glutaraldehyde achieved a 3 $\log_{10}$ reduction in 15 sec while 0.3 and 0.55% OPA achieved the same level of sporicidal activity at 2 and 1 min respectively (16). Although the mechanism of sporicidal activity of OPA has not been elucidated, the spore coat is thought to contribute to the resistance of spores. Comparison of the activity of OPA resistance of spore coat defective (UDS-treated) *B. subtilis* spores with untreated spores indicates that the treated spores are much more susceptible to OPA (2). This hypothesis has been verified (6).

Mycobactericidal activity

One of the strengths of OPA as an antimicrobial is its activity against mycobacteria when compared to that of other aldehydes. In one study, several species of *Mycobacterium* were exposed to 0.5% OPA at 20°C (4). The results indicate that under clean conditions (i.e. in the absence of organic material) at pH 8.0, there was a 5 $\log_{10}$ reduction in viable counts of *M. chelonae* NCTC 946, *M. chelonae* Epping, *M. chelonae* Harefield and *M. terrae* NCTC 10856 with a 1 min exposure. At pH 6.5 M. chelonae Epping and Harefield proved to be slightly more resistant. *M. abscessus* NCTC 10882 required 5 min and 10 min at pH 6.5 and 8.0, respectively for the same 5 $\log_{10}$ reduction. This is in contrast to the activity of glutaraldehyde at the same concentration and pH 8.0, which required extended times to achieve the same 5 $\log_{10}$ reduction. *M. abscessus*, *M. chelonae* Epping and Harefield required ≥60 min. In the presence of organic material, OPA proved to be superior to glutaraldehyde. Glyoxal, succinaldehyde and malonaldehyde proved to be ineffective against all strains at 10% concentration (4).

Working with spheroplasts of various mycobacterial strains, Fraud, Hann, Maillard et al. (5) showed that 0.5% OPA caused significant protein coagulation in *M. chelonae* NCTC 946 when compared to protein coagulation caused by 0.5% glutaraldehyde. This is most likely due to the hydrophobic nature of OPA, allowing for penetration through the lipid rich external cell layer. Glutaraldehyde being more hydrophilic is likely to act on the surface of the cells, as evidenced by the ability of glutaraldehyde treated cells to withstand osmotic shock; whereas, spheroplasts treated with OPA were sensitive to osmotic shock. Neither chemical showed significant cytoplasmic effects at concentrations below 0.5%.

Two glutaraldehyde-resistant strains, *M. chelonae* Epping and *M. chelonae* Harefield, both isolated from washer disinfectors did not show a similar resistance to OPA. Exposure of both organisms to 0.5% OPA at 25°C resulted in a greater than 5 $\log_{10}$ reduction in 10 min or less as compared to glutaraldehyde, which required >30minutes to achieve the same 5 $\log_{10}$ reduction (17). Chan-Myers and Roberts (18) also reported the rapid reduction in viable counts of a glutaraldehyde-resistant strain of *M. chelonae.* A 5$\log_{10}$

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reduction was achieved in 5 min at 25°C with OPA whereas to achieve the same effect with glutaraldehyde required 45 min.

Anti-mycobacterial antibiotics were used to elucidate the mechanism of action of OPA (17). Cells were treated with ethambutol, D-cycloserine (DC) or *m*-fluoro-DL-phenylalanine (FP), all of which exert their mode of action at the level of the cell surface. Ethambutol is thought to "open up" the mycobacterial cell wall to allow better penetration of biocides. DC and FP both inhibit the synthesis of mycoside C, one of the constituents of the mycobacterial cell wall. All three agents disrupt the cell surface allowing for better entry of chemicals into the cell. Once inside the cell these biocides can exert their activity. Ultrastructural changes in treated cells indicate that there were changes in the cell surfaces consistent showing less contiguous outer cell surface. When *M. chelonae* NCTC 946, *M. chelonae* Epping, and *M. chelonae* Harefield were pretreated with ethambutol and then exposed to 0.5% OPA or 2.0% glutaraldehyde, the OPA treated cells decreased by greater than 5 $\log_{10}$ in approximately 5 min. With glutaraldehyde treated cells, *M. chelonae* NCTC 946 achieved this reduction in 30 min while the two resistant strains of *M. chelonae* Epping and Harefield showed less than 1 $\log_{10}$ reduction in 30 min. The same pattern was seen with DC and FP treated cells. The pretreatment of cells with these anti-mycobacterial drugs supposedly increases permeability, which explains the rapid activity of OPA however it fails to explain the continued resistance of the Epping and Harefield strains of *M. chelonae* to glutaraldehyde (4).

Gregory, Schaalje, Smart, et al. (19) studied the response of several *Mycobacterium* sp. to OPA in order to determine if any of the species would be a valid replacement for the more pathogenic slow growing *Mycobacterium bovis.* The responses of rapid growing *M. chelonae* ATCC 35752, intermediate growing *Mycobacterium terrae* ATCC 15755, and slow growing *M. bovis* ATCC 35743, to two concentrations of OPA were compared in a suspension test. At the low concentration of OPA (0.05%) the response of *M. terrae* was much more resistant than *M. bovis* and *M. chelonae*, requiring 38 min versus 22min and 19 min respectively to achieve a 6 $\log_{10}$ reduction. At the higher concentration of 0.21% OPA, although some difference between the species was noted, from a practical sense there was no difference with a 6 $\log_{10}$ reduction being achieved in 2.3-6.3 min for the three organisms. In comparison to glutaraldehyde, this paper reports that with *M. bovis*, the activity of 0.21% OPA was 6 times faster than that of 1.5% glutaraldehyde. When dried on carriers, *M. terrae* NCTC 10856 and *M chelonae* NCTC 946 were shown to be much more sensitive to 0.5% OPA and 2% glutaraldehyde than *M. chelonae* Epping and Harefield, and *M. abscessus* NCTC 10882 (17). When *M. chelonae* NCIMB 1474 and *M. chelonae* Epping were exposed to in an artificial biofilm produced by suspending cells in sodium alginate and then dried onto polypropylene discs, the time required for 5 $\log_{10}$ reduction was 30 and 60 min, respectively, in the

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absence of an organic load (bovine albumin). When tested in the presence of an organic load the activity of OPA was hindered. The presence of bovine albumin is likely to hinder activity of OPA as the albumin competes with microbes as a reactant with the OPA (20).

Virucidal activity

A 0.2% solution of OPA has been shown to be effective against a wide variety of enveloped and non-enveloped viruses, including human immunodeficiency virus Type 1 in 5 min at 20°C (21). Activity against hepatitis B and C has been established using surrogate test viruses, Duck Hepatitis B virus and Bovine viral diarrhea virus, respectively with a 10 min contact time at 20°C (21, 22).

Bactericidal activity

Conventional wisdom states that biocidal agents that are effective against mycobacteria are also effective against other vegetative bacteria and fungi. In carrier tests where cells of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were dried onto glass carriers, 0.5% OPA was effective in reducing the number of viable cells by 5 $\log_{10}$ in 1 min and 6 $\log_{10}$ in 5-10 min at 25°C, comparable to the activity of 2% glutaraldehyde. At lower concentrations 0.018% OPA, the time to achieve a 5 $\log_{10}$ reduction increased to 10 min for both *S. aureus* and *P. aeruginosa*. In a suspension test at 25°C, 0.018% OPA proved to be more effective than in the carrier tests, reducing the viable population of *P. aeruginosa* by 5 $\log_{10}$ in 2 minutes (2). Clinically relevant strains of antibiotic resistant microorganisms were evaluated for resistance to OPA. Methicillin-resistant *S. aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VRE) did not show any resistance to 0.5% OPA when tested in a carrier test (23).

Studies in which *P. aeruginosa* was dried onto glass slides in an alginate mixture to create a biofilm demonstrated that 0.5% OPA was effective in reducing the number of viable organisms by $\geq 5 \log_{10}$ in 1 min and 5 min in the absence and presence of an organic load, respectively. There was an indirect relationship between the efficacy and OPA concentration in this biofilm assay, as one would expect. This same relationship also was true for efficacy and organic load (20). OPA is more efficacious against cells in suspension than cell formed as biofilms, indicating that the polymeric matrix of the biofilm may compete for OPA with the microorganisms. OPA was found to weaken biofilms of *P. aeruginosa,* which could be removed by shear stress conditions (24).

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Chapter 17

Increasing Disinfection Efficacy of Glutaraldehyde via Chemical and Physical Enhancement

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As a biocide, glutaraldehyde (GA) acts via a complex cross-linking mechanism and the 2%GA solution is one of the most popular high-level disinfectant solutions for medical instrument processing. However, it takes 4 hours to produce complete killing of 10^6 *Bacillus subtilis var. niger spores*. In this study, chemical and physical synergisms with 2%GA are evaluated for their enhancement of sporicidal efficacy, reduction of GA concentration and shortening of the use time.

In New Biocides Development; Zhu, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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Findings based on the research performed in our labouratory: (1) Complete killing of 10^6 *Bacillus subtilis var. niger spores* was achieved in 2 hours when 2%GA was used in combination with either a cationic surfactant (two examples). (2) The complete destruction of HBsAg (Hepatitis B Surface Antigen) was demonstrated when 2%GA was used in combination with a cationic surfactant within 5 minutes (vs. 30 minutes without a surfactant). (3) Complete killing of 10^6 *Bacillus subtilis var. niger spores* was achieved in 4 hours when 1%GA only was used in combination with a catatonic surfactant. The reduced GA concentration benefits health care workers for the reduced tendency of causing allergic dermatitis and eye, respiratory irritation. (4) The combined synergisms from both chemical and physical means were investigated. Thus, the 2%GA in combination with a cationic surfactant, at 40°C with ultra sonication led to the complete killing of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Mycobacterium chelonae subsp.abscessus* (all in 10^6 concentration); complete inactivation of *Poliovirus-1* [$TCID_{50}$ (Tissue Culture Infective Dose) x 10^6] and HBsAg within 5 min; killing of *Bacillus subtilis var. niger spores* in less than 10 minutes (10^3 concentration) or less than 30 minutes (10^6 concentration). (5) The above synergisms can be applied to endoscopes processing in shorter time.

Background

Glutaraldehyde (1,5-pentanedial) is a saturated dialdehyde whose molecular structure is $OHC{-}CH_2{-}CH_2{-}CH_2{-}CHO$.

The 2% glutaraldehyde (GA) is one of the most popular high-level disinfectants and chemical sterilants for medical instrument processing.

Advantages of GA include the following: excellent biocide properties; activity in the presence of organic matter; non-corrosive action on endoscopic equipment, thermometers, and metal, rubber or plastic equipment; and non-coagulation of proteinaceous material.[1]

However, it has to take 4 hours for GA to effect complete killing of 10^6 *Bacillus subtilis var. niger spores*; it has caused procititis because of GA exposure from residual endoscope's solution; keratopathy was reported to be caused by ophthalmic instruments that were inadequately rinsed after soaking in 2%GA; it is irritating to the eyes, throat, and nose of health care workers with expose of more than 0.2 ppm GA vapor; epistaxis, allergic contact dermatitis, asthma, and rhinitis have also been reported in health care workers exposed to GA.[2-6]

Objectives of this study are: enhancing sporicidal efficacy via chemical and physical synergisms; shortening the use time of disinfection and sterilization to satisfy clinical requirements; reducing the use concentration of GA to decrease exposure to GA.

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Materials and Methods

Test microorganisms

1. Vegetative forms of bacteria: *Escherichia coli (8099), Staphylococcus aureus (ATCC 6538), Pseudomonas aeruginosa (ATCC 15442).*
2. Bacterium spore: *Bacillus subtilis var.niger spore (ATCC 9372).*
3. Mycobacterium: *Mycobacterium chelonae subsp.abscessus (ATCC 93326).*
4. Viruses: *HBsAg, Poliovirus-I*

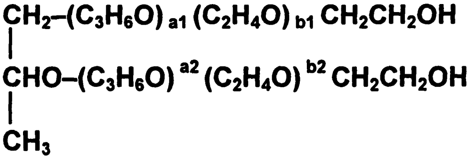
Synergisms of glutaraldehyde

1. Nonionic surfactants

A (fatty alcohol polyoxyethylene ether): $R{-}O{-}(CH_2CH_2O)_nH$

R represents a mixture of alkyl whose number of carbon is 12–15.

B (propylene glycol polyoxyethylene polyoxypropylene ether):



$$\begin{array}{l} CH_2{-}(C_3H_6O)_{a1}\,(C_2H_4O)_{b1}\,CH_2CH_2OH \\ | \\ CHO{-}(C_3H_6O)^{a2}\,(C_2H_4O)^{b2}\,CH_2CH_2OH \\ | \\ CH_3 \end{array}$$

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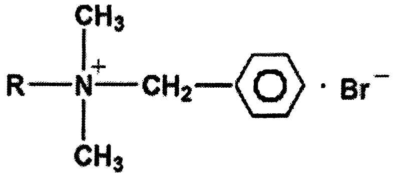
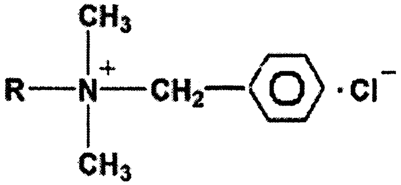
2. Cationic surfactants

C (alkyl dimethyl benzyl ammonium bromide):



R represents a mixture of alkyls whose number of carbon is 8–12.

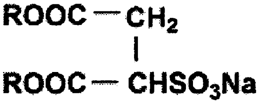
D (alkyl dimethyl benzyl ammonium chloride):



R represents a mixture of alkyls whose number of carbon is 8–12.

3. Anionic surfactants

E (sodium dialkyl ester sulfosuccinat):



$$\begin{array}{l} ROOC-CH_2 \\ \quad\quad\quad\quad\;| \\ ROOC-CHSO_3Na \end{array}$$

R represents a group of alkyls whose number of carbon is 8.

F (sodium dodecyl sulfate): $C_{12}H_{25}OSO_3Na$

4. Amphoteric surfactants

G (alkyl dimethyl betaine): $R\text{-}N^+(CH_3)_2CH_2COO^-$

R represents a mixture of alkyls whose number of carbon is 12–14.

H (sodium dodecyl sulfate + $MgCl_2$): $C_{12}H_{25}OSO_{\text{-}3}Na^+MgCl_2$

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Test method

Quantitative suspension bactericidal, virucidal and mycobactericidal tests.

Results

1. Screening synergisms of GA

Two of nonionic surfactants (A, B), two of cationic surfactants (C, D), two2 of anionic surfactants (E, F) and two amphoteric surfactants (G, H) were tested.

1.1 Complete killing of 10^6 *Bacillus subtilis var. niger spores* was achieved in 2 hours when 2%GA was used in combination with either a cationic surfactant (two examples). (Table 1)

1.2 The complete destruction of HBsAg was demonstrated when 2%GA was used in combination with a cationic surfactant (two examples) within 5 minutes (vs. 30 minutes without a surfactant). (Table 2)

2. Probing disinfection Efficacy of less than 2% GA with synergism

The reduced GA concentration benefits health care workers for the reduced tendency of causing allergic dermatitis, eye and respiratory irritation.

2.1 Complete killing of 10^6 *Bacillus subtilis var. niger spores* was achieved in 4 hours when 1% GA was used in combination with a catatonic surfactant (Table 3, Figure 1).

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Table 1. Efficacy of 2%GA in killing *B. subtilis var.niger spores* with synergism of different means.

Disinfectants (pH=6.8-7.2)	*Spore count (cfu/ml) after exposure for different periods of time (hr)*			*Compared with 2% GA*	
	0.5	1	2	X^2	P
2% GA	244000	18800	153		
2% GA + A	466000	19300	133	0.31	
2% GA + B	279000	54000	320	0	
2% GA + C	94900	1540	0	7.69	< 0.01
2% GA + D	67700	783	0	7.69	< 0.01
2% GA + E	266000	8770	557	1.23	
2% GA + F	170000	15200	1560	0.31	
2% GA + G	307000	26600	890	2.77	
2% GA + H	28600	16600	20	2.08	

NOTE: spore count of the positive control was 1110000 cfu/ml.

Table 2. Efficacy of 2%GA in destructing antigenicity of HBsAg with a cationic surfactant.

Disinfectants	*Average S/N Values after exposure for different periods of time (min)*				*S/N Values (positive control)*	*OD Values (Negative control)*
	5	10	20	30		
2% GA	4.62	3.14	2.98	1.02		
2% GA + C	1.96	1.58	1.31	0.92	126.90	0.019
2% GA + D	1.79	1.21	1.00	0.57		

NOTE: *HBsAg* was destroyed when S/N Values < 2.1.

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Table 3. Efficacy of 1%GA with D in killing *B. subtilis var.niger spores*

Disinfectants	*Average killing logarithm after exposure for different periods of time (hr)*					*Bacterial count of the positive control (cfu/ml)*
	0.5	1	2	3	4	
2% GA	2.55	3.67	4.22	5.18	> 6.58	3.80×10^6
1% GA + D	2.17	3.49	4.32	5.51	> 6.58	

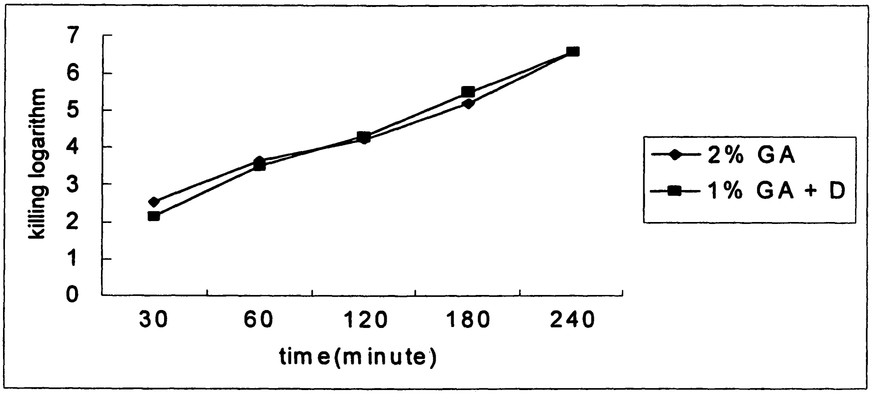

Figure 1. Efficacy of 1%GA with D in killing B. subtilis var.niger *spores*

2.2. The complete destruction of HBsAg was demonstrated when 1% GA was used in combination with a cationic surfactant D within 20 minutes (vs. 30 minutes when 2% GA was used without a surfactant) (Table 4, Figure 2).

Table 4. Efficacy of 1%GA with D in destructing antigenicity of HBsAg

Disinfectants	*Average S/N Values after exposure for different periods of time (min)*				*S/N Values of the positive control*	*OD Values of the Negative control*
	5	10	20	30		
2% GA	4.62	3.14	2.98	1.02	126.90	0.019
1% GA + D	2.63	2.32	1.98	1.33		

NOTE: HBsAg was destroyed when S/N Values < 2.1.

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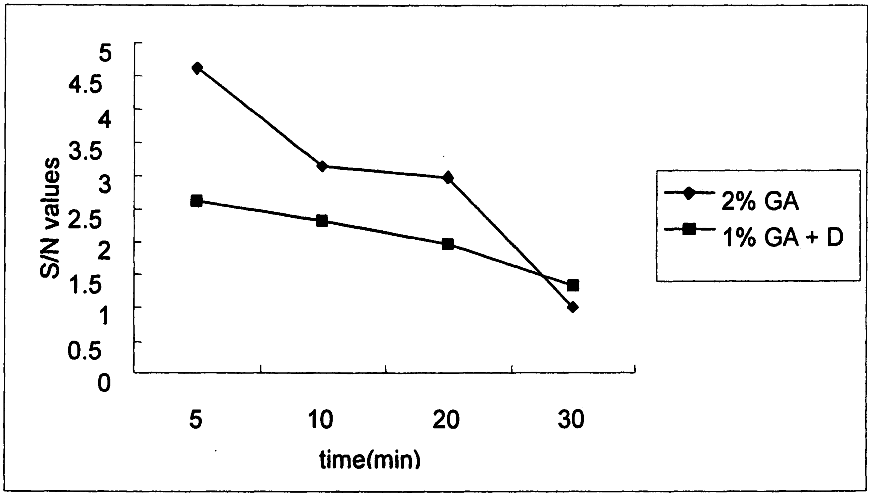

Figure 2. Efficacy of 1%GA with D in destructing antigenicity of HBsAg

2.3 Complete killing of 10^6 of *Staphylococcus aureus Pseudomonas aeruginosa*, *Escherichia coli* and *Mycobacterium chelonae subsp. abscessus* was achieved in 5 min when 0.1% GA was used in combination with cationic D (Table 5, Figure 3).

Table 5. Efficacy of 0.1% GA with D in killing vegetative forms of bacteria.

Test organisms	*Average killing logarithm after exposure for different periods of time* (min)				*Bacterial count of the positive control (cfu/ml)*
	1	2	3	5	
E. coli	2.85	3.60	> 6.10	> 6.10	1.26×10^6
S. aureus	1.87	2.60	3.80	> 6.15	1.40×10^6
P. aeruginosa	1.38	2.47	3.09	> 6.17	1.48×10^6

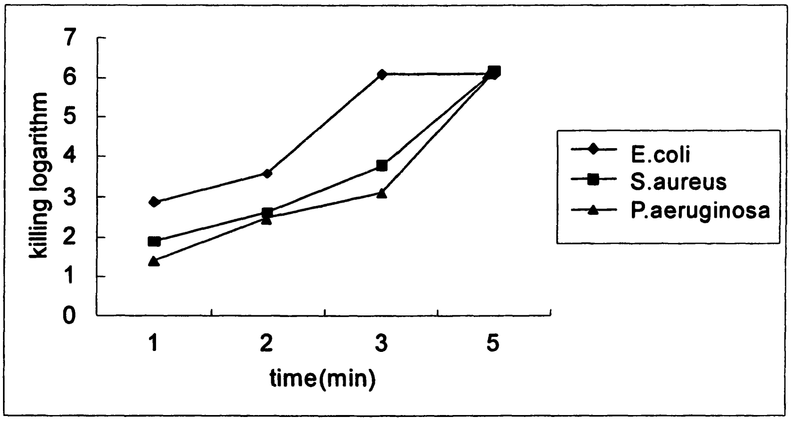

Figure 3. Efficacy of 0.1% GA with D in killing vegetative forms of bacteria

3. Researching rapid disinfection by GA

The combined synergisms from both chemical and physical means were investigated to satisfy endoscopes processing requirements.

Table 6. Efficacy of 2%GA+ D with ultrasonication at 40°C in killing Mycobacterium

Disinfectants	*Average killing logarithm after exposure for different periods of time (min)*				*Bacterial count of the positive control (cfu/ml)*
	2	5	10	20	
2% GA	4.35	6.32	> 7.26	> 7.26	1.8×10^7
2% GA + D	> 7.26	> 7.26	> 7.26	> 7.26	

2% GA in combination with a cationic surfactant D at 40°C with ultra sonication led to the complete killing of *Mycobacterium chelonae subsp.abscessus* (all in 10^6 concentration) within 2 min (Table 6, Figure 4); inactivation of *Poliovirus-1* within 1 min (Table 7); killing of *Bacillus subtilis var. niger spores* in less than 10 minutes (10^3 concentration) or less than 30 minutes (10^6 concentration) (Table 8, Figure 5).

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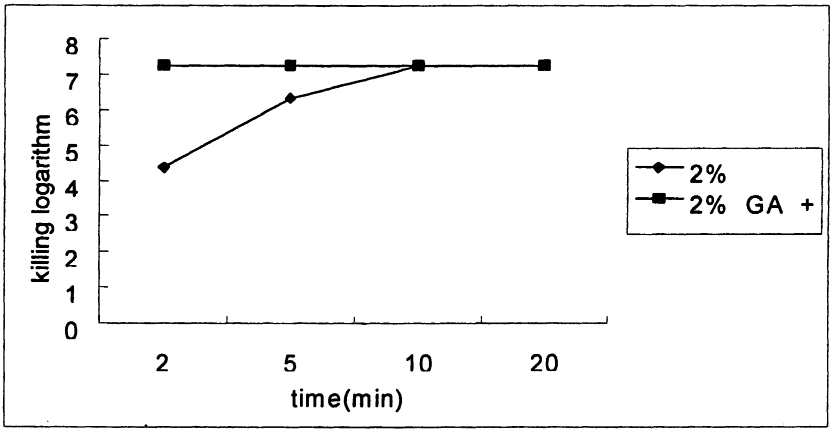

Figure 4. Efficacy of 2%GA+ D with ultrasonication at 40°C in killing Mycobacterium

Table 7. Efficacy of 2%GA+ D with ultrasonication at 40°C in inactivating Polioviruses.

Disinfectants	*Virus titer logarithm of positive control*	*Average inactivation logarithm after exposure for different periods of time(min)*		
		1	3	5
2% GA	6.75	$\geq$ 4.00	$\geq$ 4.00	$\geq$ 4.00
2% GA + D	6.75	$\geq$ 4.00	$\geq$ 4.00	$\geq$ 4.00

NOTE: Disinfection was achieved when inactivation logarithm $\geq$ 4.00.

Table 8. Efficacy of 2%GA+ D with ultra sonication at 40°C in killing B.subtilis var.niger spores

Disinfectants	*Average killing logarithm after exposure for different periods of time (min)*				*Bacterial count of the positive control (cfu/ml)*
	2	5	20	30	
2% GA	1.86	2.78	3.64	4.26	1.23×10^6
2% GA + D	2.02	3.89	5.47	>6.09	

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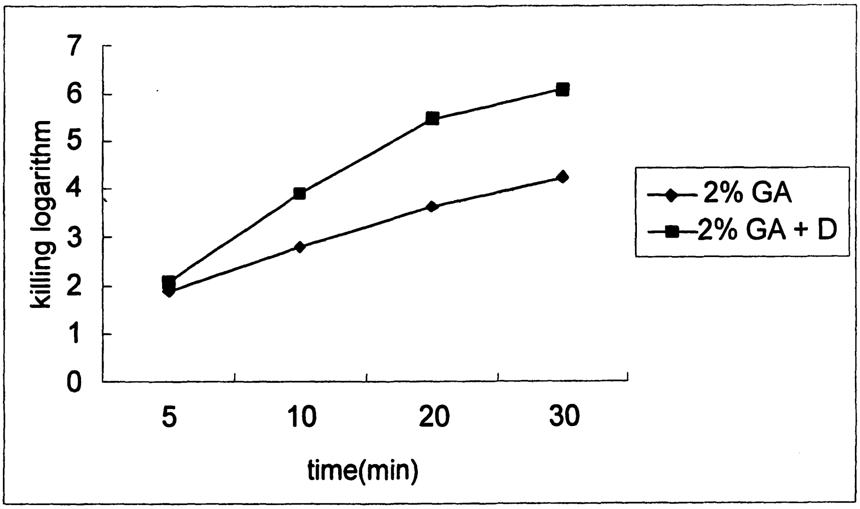

Figure 5. Efficacy of 2%GA+ D with ultra sonication at 40°C in killing B. subtilis var.niger *spores*

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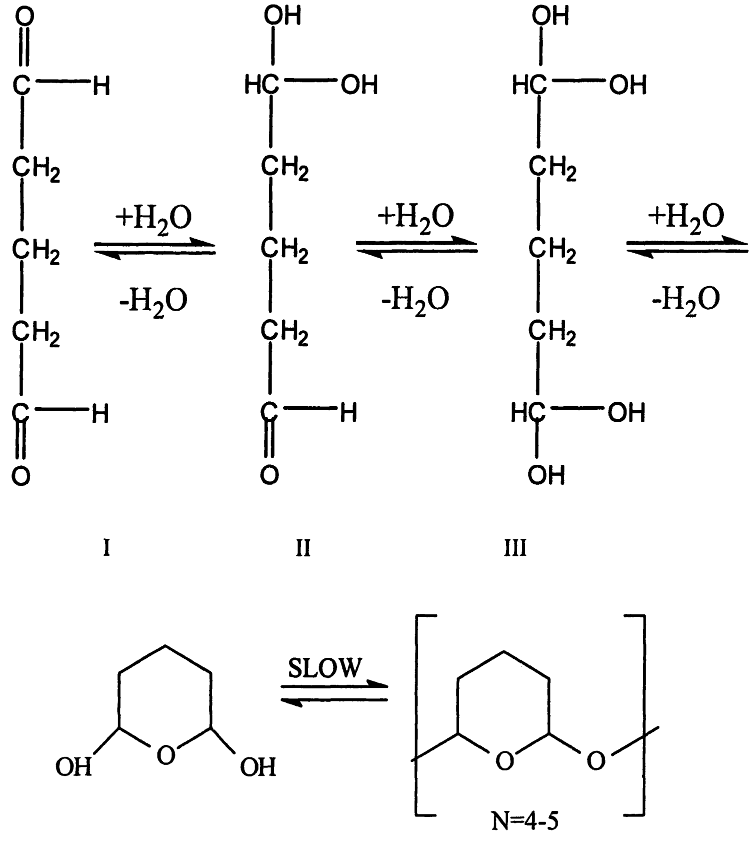
Discussion

1. Chemical properties of GA



I II III

IV V

Structurally, GA is a linear five-carbon dialdehyde. In water, GA exists in a very complex equilibrium mixture with the hydrated forms (II, III, IV, V). Five distinctly different monomeric species of GA can undergo a reversible oligomerization reaction. At four to five GA residues in length, these oligomers readily revert back to the reactive, monomeric form of GA relatively mild conditions such as dilution coupled with slight warming or a slight increase in pH.[2]

2. Microbicidal mechanism of GA

Tennen (2000) studied mechanisms of killing of spores of *Bacillus subtilis* by GA and suggested that spore killing by GA was not through DNA damage and did not cause mutagenesis; but GA inactivated the spore's germinant receptors, rendering the spore unable to respond to germinants; GA also cross-links the spores outer layers and spore sensitivity to GA was increased dramatically by removal of much spore coat protein.[7]

The productive chemistry and antimicrobial activity of GA is based on the ability of aldehydes to undergo alkylation reactions. The bifunctional nature of the GA molecule has an important consequence: each end of the molecule can chemically react with a different amino group so that the GA can form a bridge, or cross-link, between these amino groups. All organisms contain some amino functionality and are thus susceptible to attack by GA. [2]

3. The factors influencing the activity of GA

The key to the activity of GA is the fact that it is a reactive, bifunctional reagent. The factors that influence the activity of GA include pH, temperature, matrix, and the nature of the use system.

Aqueous solutions of GA are acidic and generally in this state are not sporicidal. Only when the solution is "activated" (made alkaline) by alkalizing agents to a pH of 7.0 to 8.5 does the solution become sporicidal.

Temperature influences the concentration of free aldehyde. Free GA is 33 % at 50°C while free GA is 15 % at 25°C. [8]

Wang Chuan et al (2004) studied the synergetic germicidal effect of ultrasound with GA and found the concentration of GA needed for sterilize spores was decreased from 2% to 0.5% for the same killing time (4h) and the killing time was shortened from 4h to 1h when the concentration were still 2% because of the synergetic germicidal effect. [9]

Surfactants reduce the surface tension of cells so that disinfectants can rapidly attack into microorganisms and increase the rate of activity of GA. Studies of Shen Wei et al (1996, 1997) and Yin Tao et al (2005) have indicated the synergetic germicidal effect of cationic surfactants with GA. Future research will prove that synergisms to GA of cationic surfactants are more than others.

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Chapter 18

Natural Antimicrobials from Plant Essential Oils

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Plant essential oils are volatile compounds that have been widely used in perfumery, aromatherapy, cosmetics, and for flavoring food and drink, and to a lesser extent, been used in food preservation, in medicine and household cleaning products. Essential oils and their major components are generally recognized as safe (GRAS), and because of their diverse biological activities, are focus of many studies looking for alternative agents to control bacteria, fungi and viruses in foods, crops, humans and animals. Monoterpenoids and phenylpropanoids are the major and perhaps the most important components of the various essential oils. These natural products have been proven to be a good source of antibacterial agents, particularly against food-borne pathogenic bacteria such as *Escherichia coli*, and *Salmonella typhimurium*. Essential oils or their major components have the potential to play an important role in food safety. They may also be used to control fungal decay of food, extending the shelf life of fresh produce and contributing to safer food by inhibiting the growth and mycotoxins production of important food-, feed- and soil-borne plant fungal pathogens. The antiviral and antifungal activity of certain essential oils and their components against human diseases provide a safe alternative to the synthetic drugs, particularly in immunocompromised individuals.

In New Biocides Development; Zhu, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Introduction

Essential oils (EOs) are concentrated, often hydrophobic liquids containing volatile aromatic compounds extracted from plants. They are normally responsible for the distinctive aromatic odor or smell of different plants or plant parts. Although the term essential is intended to indicate that the oil is the fragrant essence of the plant from which it is extracted and not in the more common sense of being indispensable, many of the EOs or their components have been found to be important for the plants' self-defense against invading insects or microorganisms. EOs are used in perfumery, aromatherapy, cosmetics, incense, for flavoring food and drink, and to a lesser extent, in medicine and household cleaning products. They may be produced by distillation, expression, solvent extraction, or newer technologies such as supercritical fluid extraction. They are valuable commodities to the fragrance and flavorant industries.

Components of the EOs have been used since antiquity in food preparation both for their flavor and for their preservative properties (*1*). A number of EO components are generally recognized as safe (GRAS) by the FDA (Food and Drug Administration) of the United States and have been used as artificial flavorings, in the manufacture of perfume (*2*) and in over-the-counter formulations of medicines (*3*). EOs and its major components, monoterpenoids, are known to be the chemical defense against insects, fungi and other invaders (*4*). Studies have also shown strong antimicrobial activity in certain EO components (*5-8*) and the potential use of EOs to control postharvest decay has been examined for fruits, vegetables and flowers (*9-13*).

In recent years studies have been done to investigate the antimicrobial compounds in EOs of different plants and their roles in food safety and human and animal health. It this chapter, we provide a summary of the chemistry of EOs and their bioactive components, and how they can be used to control plant- and food-borne diseases, and as natural antimicrobial agents against human and animal diseases.

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Chemistry and Biochemistry of EOs

EOs and Biosynthesis

Chemically, the constituents of plant EOs fall mainly into two entirely distinct chemical classes, terpenoids and phenylpropanoids (*14*) (Figure 1). Many small molecules belonging to different chemical categories including aldehydes, ketones, alcohols and esters, are found in plant EOs. Although terpenes, particularly monoterpenoids, occurring much more frequently and abundantly represent the major EO components, phenylpropanoids provide indispensable and significant flavor and odor to the oils (*14*).

In plants, terpenoids and phenylpropanoids are synthesized from different primary metabolic precursors and through different biosynthetic pathways. In volatile oil plants, the biosynthesis of terpenoids has been much more widely investigated and regularly reviewed (*15*). Phenylpropanoids originate through the shikimate pathway. The biochemical mechanisms of oil-specific phenylpropanoid synthesis, such as eugenol, are known only to a very limited extent. The following is a brief summary on the major biosynthetic pathways of EO components. Readers are referred to the latest review on the subject for a detailed description (*16*).

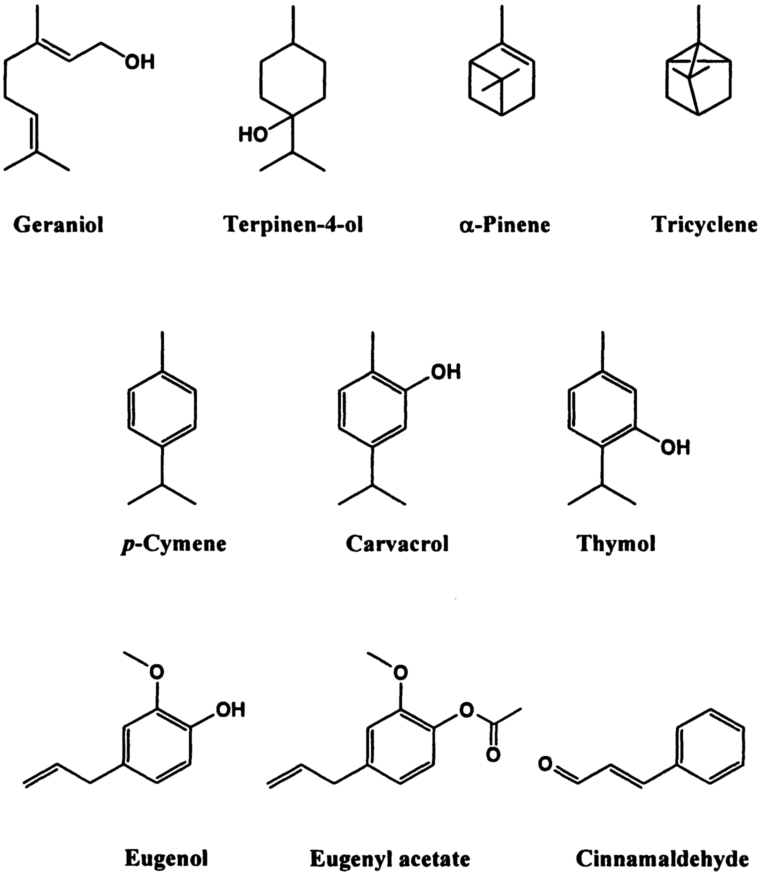

Figure 1. Typical EO components. Top panel: different structural features (linear, mono-, bi- and tricyclic monoterpenoids); Middle panel: aromatic and phenolic monoterpenoids; Top and Middle panels: monoterpenoids; Bottom panel: phenylpropanoids.

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Monoterpenoids are the largest and most diverse class of organic compounds found in plants EOs (*13*). Chemically, these compounds are defined as C10 compounds based on two isoprene (C_5H_8) units connecting in a head-to-tail fashion (*17*), but their structural features can be in acyclic, monocyclic, bicyclic and tricyclic forms (Figure 1). Monoterpenoids are synthesized in various cellular organelles, and stored in specialized secretory structures for the protection of the plants' metabolic processes from their own phytotoxic effects (*18, 19*). Isoprene molecules are synthesized from three acetyl-CoA molecules which form the six-carbon intermediate, 3-hydroxy-3-methylglutaryl-CoA, which is then reduced to mevalonic acid (*18*). From mevalonic acid, IPP (isopentenyl pyrophosphate) is formed after phosphorylation and decarboxylative elimination. IPP is also isomerized to DMAPP (dimethylallyl pyrophosphate). IPP and DMAPP are the building blocks of all terpenoids including monoterpenoids, and the head-to-tail combination of these two compounds gives GPP (geranyl pyrophosphate), the most important intermediate leading to the creation of the 1500 monoterpenoids in plants (*13*).

The phenylpropanoids are less common than monoterpenoids but, when present, contribute significantly to the sensory value of the oil. EO components in this category have the phenylpropene skeleton which is derived from phenylalanine and synthesized via the shikimate pathway (*14*). One of the most recognized EO component, eugenol is produced from this pathway (*14*).

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Factors Affecting the Production of EOs in Plants

The composition and yield of plant EOs can be affected by various factors including plant ontogeny, light, growing season, soil nutrition, environmental conditions (e.g. moisture, salinity and temperature), and the use of plant growth regulator. It is well known that plants of the same species have different chemotypes due to genetic variations. For the same species, plant ontogeny is one of the most important characteristics of oil accumulation in plants. EO production in most plants largely depends on the developmental stage/phase of the plant well as its concerned part/organ, tissue and the cells (*14*). Most studies conducted thus far indicate that net EO production is associated with the early growth period, such as in *Mentha piperita* (*20*). The oil composition also changes significantly at different growth stages, for example, in *Cympopogon flexuosus*, geraniol increases from 65% to 81% until flowering stage, and has formed at the expense of geranyl acetate (*14*). The effect of growth phase may also depend on the type of EO components. For example, in a recent study, Oliveira *et al.* (*21*) found that the phase of growth did not affect the production of monoterpene hydrocarbons in *Hyptis suaveolens*.

The production of EOs, as with most plant-derived phytochemicals, depends largely on the length and intensity of light, particularly for plants where vegetative proliferation and foliage biomass are usually the main considerations for the 'oil harvest'. Photoperiod may exert its influence through modulations of the plant's relevant metabolic functions to alter the yield and composition of EO components. Light of different electromagnetic wavelengths also affects biosynthesis of EO, in particular, red light enhanced the biogenesis of EO (*14, 22, 23*). Sage plants grown at full or 45% of full sunlight differed in monoterpenoid composition in the EO (*24*).

Environmental conditions such as atmospheric temperature and rainfall have been reported to influence oil content and composition in several aromatic plants. An increase in temperature reduced oil content but increased percentage of citronellol and its formate ester in the oil of a chemotype of geranium, whilst rainfall increased the amount of geraniol in another chemotype (*25*). Seasonal patterns of oil accumulation and composition recorded over two years in *Artemisia dracunculus* revealed that the accumulation of oil was greatest in July (*26*). Three chemotypes of *Thymus piperella* L. responded differently to environmental factors such as soil acidity, water balance and organic matter (*27*). Fertilizer applications generally affect oil yield by enhancing the amount of biomass yield per unit area, (*28*). Manganese was the most effective single micronutrient enhancing oil production, while other minerals may have no or little effect (*29*). Soil mineral contents also affect the yield of both biomass and EO. Oliveira *et al.* (*21*) found that monoterpene hydrocarbons were mainly produced in plants located in higher latitudes and altitudes regardless of the phase of growth, while sesquiterpenes were mainly produced in fruiting samples grown at lower sites. Their study also revealed a significant relationship between oil components and edaphic (soil) factors (*30*). A study on the leaf EOs of *Cistus monspeliensis* plants grown on two types of soils in Southern France showed significant qualitative and quantitative differences in the EO composition (*31*). A recent study has shown that heavy metals such as Cd, Pb, and Cu may slightly negatively affect the yields and EO compositions of peppermint, basil and dill (*32*).

Processing method such as the drying conditions before the extraction is also important. Plants dried by three different methods (sun-drying, shade-drying and oven-drying at 45 °C) were compared. No significant difference was found between oil yield (w/w) of the oven-dried sample (1.06%) compared to shade-dried (0.94%) and sun-dried (0.87%). However, the oil content of the shade-dried sample, obtained by hydro-distillation, was higher (0.94%) than that of the steam-distilled (0.27%) (*33*).

Isolation, Quantitation and Structure Identification

Isolation techniques for plant Eos have been found to affect the chemical composition and bioactivity of the oil. Extraction of EOs can be done by three methods: distillation, expression and solvent extraction. There are two types of

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distillation, hydrodistillation (HD) and steam distillation. Expression or cold-pressed means that the oils are expressed mechanically. Solvent extraction uses either organic solvents such as ethanol and hexane, or supercritical fluid such as carbon dioxide. EOs and their major components are relatively lipophilic and are therefore readily extracted by organic solvents such as acetone, dichloromethane or hexane, depending on their lipophilicity. Many of the oxygenated monoterpenoids, however, are water soluble at biologically active concentrations (*12, 34*).

The most commonly employed methods for the isolation of EOs are steam distillation and solvent extraction. However, the problem with steam distillation is that it can cause thermal isomerization and loss of volatiles of very low boiling point (*13, 35*). These disadvantages can be minimized by direct solvent extraction and vacuum distillation (molecular distillation). One of the latest technologies for EO extraction is supercritical fluid extraction (SFE). When using CO_2 as the extraction solvent in SFE, components that are very volatile can be contained, resulting in not only high efficiency and purity of the extracts, but also less adverse environmental impact (*36-38*).

Separation and purification of EO components such as monoterpenoids involves mostly chromatographic techniques (*18*). Multiple steps are usually involved in the separation, including liquid-liquid partitioning, normal phase (silica gel) or reverse phase (C8 or C18) column chromatography, preparative thin layer chromatography (TLC), fractional crystallization and re-crystallization.

Only newer extraction technologies will be briefly discussed in this chapter. Fadel *et al.* (*39*) studied the volatile oil compositions of *Eucalyptus camaldulensis* var. *brevirostris* leaves obtained by HD and SFE methods. They found that in both extracts the main constituents were ß-phellandrene (8.94 and 4.09%), *p*–cymene (24.01 and 10.61%), cryptone (12.71 and 9.82%) and spathulenol (14.43 and 13.14%). The yield of the monoterpene hydrocarbons in HD oil was slightly higher compared with that in the SFE extract (*39*). The SFE extract also contained higher concentrations of the sesquiterpenes, light-oxygenated compounds and heavy-oxygenated compounds than the HD oil (*39*).

A microwave assisted ethanol extraction (MAE) and a 2-h HD technique were compared in terms of the total EO yield in dry and fresh tea tree (*Melaleuca alternifolia*) leaves. There was no significant compositional difference between dry and fresh leaf, however, the HD technique recovered less EOs than MAE. For both samples the HD oil also consisted of lower amounts of sesquiterpenoids and marginally lower amounts of monoterpenoids. Extending the HD distillation to 6 h increased the sesquiterpenoid recovery but this resulted in a reduction in both the absolute and relative amounts of the oxygenated monoterpenoids, terpinen-4-ol and 1,8-cineole (*40*)

EOs of clove buds obtained by SFE, hydrodistillation, steam distillation and Soxhlet extraction were further analyzed and compared by Guan *et al.* (*41*).

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Twenty three compounds in the clove oils were identified, showing that the composition of the clove oils extracted by different methods were mostly similar, whereas relative concentration of the identified compounds were apparently different. They concluded that in general SFE was an ideal method among the four processes for obtaining clove oil with high yield and high quality.

Solvent-free microwave extraction (SFME) is a recently developed green technique which is performed in atmospheric conditions without adding any solvent or water (*42*). A kind of microwave absorption solid medium, such as carbonyl iron powders (CIP), was added and mixed with the sample before extraction. SFME can be applied to extraction of EO from the dried plant materials without any pretreatment. Because the microwave absorption capacity of CIP is much better than that of water, the extraction time while using the improved SFME is no more than 30 min using a microwave power of 85W. Compared to conventional SFE, the advantages of improved SFME were to speed up the extraction rate and need no pretreatment. Improved SFME has been compared with conventional SFE, microwave-assisted hydrodistillation (MAHD) and conventional HD for the extraction of EO from dried *Cuminum cyminum* L. and *Zanthoxylum bungeanum* Maxim. There was no obvious difference in the quality of EOs obtained by the four extraction methods (*42*). In general, methods used to extract EO from plants depend on types of components. SFE seems better for sesquiterpenoids, while HD may be more efficient for monoterpenoids. MAE is a good method for monoterpenoids.

Analytical separation and detection of EOs and their components can be done by TLC, gas chromatography (GC) or high performance liquid chromatography (HPLC), depending on their volatility, structure (double bonds etc.) and other functional groups. GC and HPLC, particularly GC are widely used in quantitative analysis (*18*). Structures of unknown compounds are elucidated using nuclear magnetic resonance (NMR), infrared spectroscopy (IR), mass spectrometry (MS) and ultraviolet spectroscopy (UV). Hyphenated technologies such as GC-MS and HPLC-MS are also very useful in structural identification (*13*). Monoterpenoids and other EO components are separated through the GC or HPLC system and detected by the MS detectors. The former generates the retention time for a compound and the latter a mass spectrum; both of which can be compared with standards. The MS detector can be used simultaneously used with other detectors such as IR in GC-IR, and UV in HPLC-UV. The photodiode array detector in HPLC provides UV spectral information that is valuable for structure identification (*13*).

Biological Activity of EOs

EOs and their components, like many other naturally-occurring phytochemicals, play important biological roles as ecologically active compounds.

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Such biological roles are played additively or synergistically among the different compositions of a specific oil or between different oils (*19*). Another important observation with these compounds is that a single EO component may have multiple biological effects (*19*). They can have direct effects as plant chemical defense mechanisms against invading herbivores, microbial infections and other plants as competitors for nutrients. The effects may also be indirect, for they can be attractants for enemies of insect herbivores or pollinators. Therefore EOs can have a broad spectrum of activity against other organisms, but that activity can also be very specific in terms of dosage levels on different populations of interacting organisms in different communities (*19*). This section discusses recent findings of the antimicrobial activities of EOs, i.e. the antibacterial, antifungal and antiviral properties.

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Antibacterial Activities

Food safety is an increasingly important public health issue despite the significant modern improvements in slaughter hygiene and food production techniques (*43*). It has been estimated that as many as 30% of people in industrialized countries suffer from a food-borne disease each year and in 2000 at least two million people died from diarrhoeal disease worldwide (*44*). Therefore, a need exists for new methods of reducing or eliminating food-borne pathogens, possibly in combination with existing methods (*45*).

Spices and herbs rich in EOs have been used for their flavor and preservative properties since antiquity (*46*). However, the use of extracted EOs did not happen until 2000 years ago when distillation was invented (*43*). EOs and their principal components possess antibacterial activities against many food-borne and non-food-borne bacteria, and have been used commercially for dental products to food preservatives, and in feed supplements for animal production (*43*).

Methods for *in vitro* evaluation of the EOs against different bacteria, and their pros and cons, have been reviewed recently (*43*). In brief, screening of EOs for antibacterial activity is often done by the disk diffusion method, and the strength of the antibacterial activity can be determined by dilution of EO in agar or broth. In the latter technique, optical density (OD) measurement and the enumeration of colonies by viable count are the most often used end-points (*43*).

Common food-borne bacteria include *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus*. Many EOs effectively inhibit the growth of these bacteria; among them, EOs containing phenolic components are particularly of interest because they have consistently stronger antibacterial activities with lower minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). Table I lists the MICs of three most effective EOs of clove, oregano and thyme against these bacteria.

Table I. MICs (µg/mL) of Three Most Effective Antibacterial EOs. [a]

Food-borne Bacteria	*Clove*	*Oregano*	*Thyme*
Escherichia coli	0.4–2.5	0.5–1.2	0.45–1.25
Salmonella typhimurium	>20	1.2	0.45–>20
Staphylococcus aureus	0.4–2.5	0.5–1.2	0.2–2.5
Listeria monocytogenes	0.3	-	0.16–0.45

[a] Data were abstracted from Table 4 of Reference 43 with permission.

The principal component of clove EO is eugenol (85%) as its major component (*47*). Oregano, particularly the Greek oregano, is known to be the best source of carvacrol. Depending on the harvest season and location, the EO of Greek oregano may contain up to 93% carvacrol (*48*). EO from the leaves of thyme can contain up to 52.4% thymol (*49*). The antibacterial activities of the EOs of clove, oregano and thyme have been largely attributed to their respective major components, eugenol, carvacrol and thymol, all of which are characteristically phenols. This has been confirmed by testing the antibacterial activities of these three phenols (Table II). The modes of action of the antibacterial activities of EO components are not always clear, and may differ depending on the molecular structures. However, it is generally accepted that compounds such as eugenol, thymol and carvacrol, due to the hydroxyl group in the phenolic structure, bind to enzymes or membrane proteins hydrophobically and by means of hydrogen bonding, thereby changing the permeability characteristics of the membrane or preventing enzyme action (*50-52*). Most recently, we found that such binding by carvacrol or thymol may also affect the cell division of common food-borne bacteria (*8*).

Table II. MICs (µg/mL) of Three Phenolic Antibacterial EO Components [a]

Food-borne Bacteria	*Eugenol*	*Carvacrol*	*Thymol*
Escherichia coli	1.0	0.23-5.0	0.23-0.45
Salmonella typhimurium	0.5	0.23-0.25	0.06
Staphylococcus aureus	-	0.17-0.45	0.14-0.23
Listeria monocytogenes	>1.0	0.38-5.0	0.45
Bacillus cereus	-	0.19-0.90	0.45

[a] Data were abstracted from Table 5 of Reference 43 with permission.

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Major components alone may not explain the total antibacterial activity for certain EOs. The synergistic or additive effects from minor components in the EO may be significant (*53, 54*). Such synergism was also observed at the individual component level; for example, carvacrol and thymol, both found in oregano EO, had an additive effect (*50*).

The strong *in vitro* antibacterial activities of EOs or their major components have led to many applications in non-food products such as cosmetics. The strong flavor, volatility and lipophilicity of most of the EOs have limited their use as food preservatives. To date, applications have been mostly in foods with strong flavors such as sausages and cheese. Beside these technical difficulties, a greater concentration of EOs is needed in foods to achieve the same efficacy as that found in antibacterial assays *in vitro* (*55, 56*). The magnitude of concentration increase can be from two-fold higher in certain foods such as milk (*57*), to 100-fold higher in soft cheese product (*58*). The phenomenon has not been well explained. The complex food matrix (fat/protein/water content, antioxidants, preservatives, pH, salt and other additives) the extrinsic determinants (temperature, packaging in vacuum/gas/air, characteristics of microorganisms), the greater availability of nutrients in foods compared to laboratory media may have hindered the action of EOs and their active components, and may have enabled bacteria to repair damaged cells faster (*43, 53*).

In addition to the antibacterial activity, EOs are also strong antioxidants, and this is a great advantage for them to be used as food preservatives since they not only prevent food from microbial spoilage, but also from oxidation of fatty acids (*59*).

Antifungal EOs and Their Applications

Antifungal Activities Against Plant Pathogens

Plant pathogenic fungi are the major causes of plant diseases. Fungi can attack plants either during growth or after being harvested and stored. In agricultural crops, fungal diseases often cause severe yield losses and serious quality damage. Many synthetic chemicals have provided protection of the crops from fungal infections. Frequent applications and often high doses, however have resulted in fungal resistance and increased chemical residues in our food supply and environment. Plant EOs play a role in plant defence mechanisms against plant pathogenic fungi. Therefore, they have been studied as alternative antifungal agents against major plant fungal pathogens, and evaluated for their ability to protect crops from fungal infections (*60, 61*). Several EOs-based products such as Cinnamite™ and Valero™ developed by

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Mycotech Corporation from cinnamon oil, have already been registered for commercial uses in horticultural crops and grapes (*62*). TALENT, a product based on carvone, a monoterpene isolated from the EO of *Carum carvi*, has been registered in Europe for inhibiting potato sprouting and protecting potato tubers from fungal spoilage during storage (*61*).

Due to their low safety concerns, EOs have been intensively studied for their potential applications in the control of postharvest decay of fruits in recent years. The major postharvest losses of fruit are fruit decay caused by fungal species of *Botrytis, Penicillium, Monilinia, Alternaria, Rhizopus,* and *Colletorichum*, growth of which are inhibited by certain EOs *in vitro* (*63-65*). However, results from *in vitro* tests are not always applicable in *in vivo* situations (*66*), and only a few EOs have been reported thus far to protect stored fruit from fungal spoilages under *in vivo* conditions.

Tsao and Zhou (*67*) tested several EO components, including carvacrol, citral, citronellol, eugenol, perillalcohol, perillaldehyde and thymol, and discovered that dipping sweet cherries in carvacrol or thymol solutions at 1000 ppm for 4 min could significantly reduce brown rot of cherries caused by *Monilinia fructicola*. In another test (*12*) thymol and carvacrol also showed the most potent fungicidal activity against *Botrytis cinerea*, and significantly controlled grey mold of strawberries, with higher efficacy than the fungicide iprodione. Carvacrol, oregano, clove and cinnamon EOs were the best inhibitors of mycelial growth of *Penicillium* species, however, they were ineffective in controlling blue mold of pear (*66*) and citrus (*60*).

EOs can be applied as fumigants for stored products since their activity is in the vapor phase. Sweet cherry fruits fumigated with thymol at 10 mg/L space significantly reduced the incidence of brown rot (*68*). Thymol fumigation was also effective in controlling brown rot of apricot and plum (*69*). Thymol in vapor form also reduced fruit rot of sweet cherries in modified atmosphere packaging (*70*).

EOs have also been used in postharvest storage of vegetables. Carvone, a monoterpenoid, inhibited both sprouting and spoilage of potato tubers during storage (*61*). EO extracts from pepper fruit (*Dennetia tripetala*) had antifungal activities against pathogens such as *Rhizopus stolonifer*, *Aspergillus niger* and *Fusarium* sp. isolated from spoilt tomato fruits at concentrations of 2.5-6.5 and 1.5-3.0 mg/mL tomato or glucose medium, respectively (*71*). Tomato fruits treated with this EO extract combined with mild heat as a preservative hurdle were free of these fungi after one month of storage.

Plant-derived EOs also possess strong antifungal activities against soil-borne plant pathogens. Soylu *et al.* (*72*) investigated the antifungal activities of EOs obtained from aerial parts of aromatic plants such as oregano, thyme, lavender, rosemary, fennel, and laurel against *Phytophthora infestans*, a cause of

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late blight disease of tomato. EOs of thyme, oregano, rosemary, lavender, fennel and laurel in this study contained carvacrol (37.9%), carvacrol (79.8%), borneol (20.4%), camphor (20.2%), anethole (82.8%) and 1,8-cineole (35.5%), respectively, as their major component. All EOs were found to inhibit the growth of *P. infestans*. In addition, it was noted that EOs in volatile phase were consistently more effective in inhibiting fungal growth than those by contact (*72*).

Antifungal Activities Against Food-Borne Fungi

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One of the major problems facing the food industry is spoilage caused by microorganisms that reduce the shelf life and cause irremediably damage to food. Food spoilage fungi can be divided into yeasts and moulds, both of which are able to grow in almost all food products. The most important feature of moulds from a food safety perspective is their ability to produce mycotoxins, such as aflatoxins, ochratoxin A (OTA), patulin and vomintoxin (DON). Mycotoxin-producing fungi associated with food mainly belong to the genera *Aspergillus, Fusarium*, and *Penicillum*, and many plant EOs possess antifungal activity towards these moulds (*73, 74*).

Rusul and Marth (*74*) reviewed earlier researches on the antifungal activities of EOs against the toxigenic Aspergilli, and concluded that eugenol from the EO of clove and thymol from thyme EO, could completely inhibit the growth of *Aspergillus flavus* and *Aspergillus versicolor* at < 4 mg/mL. Thymol also totally suppressed the growth of *A. parasiticus* at 500 ppm (*74*). Recently, Nguefack *et al.* (*75*) investigated five EOs from *Cymbopogon citratus*, *Monodora myristica*, *Ocimum gratissimum*, *Thymus vulgaris* and *Zingiber officinale*, and examined their effect against three food spoilage and mycotoxin-producing fungi, *Fusarium moniliforme*, *Aspergillus flavus* and *Aspergillus fumigatus*. They found that EOs from *O. gratissimum*, *T. vulgaris* and *C. citratus* were the most effective in inhibiting both conidial germination and growth of all three fungi at 800, 1000 and 1200 ppm, respectively. Similarly, among the EOs from 12 medicinal plants, the oils of thyme and cinnamon (<500 ppm), marigold (<2000 ppm), spearmint, basil, quyssum (3000 ppm) were found to completely inhibit the growth of *A. flavus*, *A. parasiticus*, *A. ochraceus* and *F. moniliforme* (*76*). The extent of inhibition of fungal growth and mycotoxin production was dependent on the concentration of EOs used (*76*).

Some EOs may be more effective in reducing mycotoxin production than in suppressing the growth of the fungi that produce the mycotoxins. Bullerman (*77*) studied the correlation between the inhibition of mycelial growth and reduction of aflatoxin production of *A. parasiticus* in the presence of different concentrations of cinnamon EO, and found that cinnamon EO was more inhibitory to toxin production than to mycelial growth. Paranagama *et al.* (*78*) found that EO from lemongrass was fungistatic and fungicidal against *Aspergillus flavus* at 600 and 1,000 ppm, respectively. However, at a non-inhibitory low concentration of 100 ppm, aflatoxin production by this fungus was completely inhibited. Such a phenomenon was more obvious in an earlier study by Paster (*79*) who observed that EOs of cinnamon and black pepper inhibited aflatoxin formation even without inhibiting the mycelial growth (*79*).

Environmental conditions under which the fungi grow may affect the outcome of essential oil treatment for mycotoxin production. In a study by Velluti *et al.* (*80*), EOs of cinnamon, clove, lemon grass, oregano and palmarosa inhibited the growth of *Fusarium verticillioides* isolates under all four environmental conditions tested, but fumonisin B1 (FB1) was only produced by the fungus under two conditions. Soliman and Badeaa (*76*) showed that the EOs of thyme, cinnamon, anise and spearmint affected the fungal development and the subsequent mycotoxin production in wheat grains. Neem extract (*81*) and the EOs of *Cinnamomum zeylanicum* and *C. nardus* (*82*) also reduced aflatoxin production in *A. parasiticus* and *A. flavus*. The reasons behind these varied observations are not clear. EO treatment might have affected the biosyntheses of the different mycotoxins in different mycotoxin-producing fungi.

The inhibitory effect of EOs on fungal growth and mycotoxin production can vary significantly depending on their chemical compositions which can be affected by many factors as we discussed earlier in this chapter. EO compositions may differ among different species of the same genus and even within the samples of the same species. Rasooli and Abyaneh (*83*) compared EOs extracted from two varieties of thyme (i.e. *Thymus eriocalyx* and *Thymus x-porlock*) and found that the oils from both varieties were fungicidal to *A. parasiticus* and inhibitory to aflatoxin production. The profile of the oil components from *T. eriocalyx* was similar to that of *T. x-porlock* in almost all the compounds but at different concentrations. The major components of *T. eriocalyx* and *T. x-porlock* oils, thymol, β-phellandrene and *cis*-sabinene hydroxide, were 64.3% and 30.7%, 13.2% and 39.4%, 8.4% and 9.7%, respectively. The intra specific variability of oils in the *Thymus* genus has been the subject of several studies reviewed by Stahl-Biskup (*84*).

EOs used for the Control of Human Fungal Diseases

Three groups of fungi are known to cause human diseases – filamentous forms, single cells (yeasts) and dimorphic fungi that have certain growth characteristics of both. Fungal infections in humans tend to be chronic, often require prolonged chemotherapy and present particular risks for immuno-compromised individuals. The most common fungal infections are confined to

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skin, mucous membranes and nails and are often difficult to eradicate with topical preparations and may require long-term use of systemic drugs. EOs have long been used as traditional medicines, and have been the focus of recent studies on alternative antifungal drugs.

Many studies on EOs have targeted *Candida* species, particularly, *C. albicans*, which causes mild to severe chronic superficial infection of skin, nails, and membranes in normal individuals and serious systemic infection debilitated patients. Pauli (*85*) has published a very thorough review on anti-candidal compounds in EOs. The review summarized active anti-candidal compounds according to testing methods with which their inhibitory activities were identified. For example, thirty EO compounds had a Minimal Inhibitory Amount (MIA) of 10 μg or less on TLC plates in the bioautography test; forty-eight had the Minimal Inhibition Concentration (MIC) lower than 150 μg/mL in the agar dilution test. The compounds with anti-candidal activity belonged to 10 chemical groups: aliphatic compounds, monoterpenoids, sesquiterpenoids, diterpenoids, quinoids, N-heterocyclics, flavonoids, sulfur-compounds, phenylpropanoids and aromatic compounds. Tampieri *et al.* (*86*) evaluated sixteen commercial EOs and forty-two pure EO constituents against a strain of *Candida albicans* and found that the EOs of *Origanum vulgare*, *Satureja montana*, *Mentha piperita*, *Cinnamomum verum*, *Cymbopogon flexuosus* showed MICs at 500 ppm. In terms of pure constituents, β-phellandrene was the most interesting component among cyclic monoterpenic hydrocarbons as it showed a strong activity (MIC = 50 ppm). The most active phenolic compound was carvacrol (MIC=100 ppm). The open-chain alcohol 1-decanol was the most active of alcohols with an MIC at 50 ppm, and among aldehydes, a strong activity was shown by *trans*-cinnamaldehyde (MIC=50 ppm). Other EOs worth mentioning included allicin from garlic, which possesses promising efficacy against *C. albicans* and was less toxic than synthetic antifungal drugs such as amphotericin B (*85*) and, EOs from *Thymus* species (*87, 88*).

Aspergillus species is the second most common fungus in opportunistic mycoses following *Candida*, and can cause invasive infections which are associated with high morbidity and mortality, particularly in immunocompromised patients. In a report by Pawer and Thaker (*89*), 75 different EOs were compared for their inhibition of hyphal growth and spore formation in *Aspergillus niger*, an opportunistic human pathogen and a strong air pollutant. EOs of *Cinnamomum zeylanicum* (bark), *Cinnamomum zeylanicum* (leaf), *Cinnamomum cassia*, *Syzygium aromaticum* and *Cymbopogon citrates* were among the most effective against this fungus (*89*). The EO from tea tree, which has been well studied for its activity in controlling human fungal infections, was also found to be effective against *A. niger* (*90*).

Another fungal group important to humans is the dermatophytes, which can cause infections of the skin, hair, and nails due to their ability to utilize keratin.

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The organisms colonize the keratin tissues and cause inflammation in the host as a response to the metabolic by-products of this fungus. These infections are known as ringworm or tinea, in association with the infected body part. The common dermatophytes may include *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Epidermophyton floccosum*, and *Epidermophyton stockdale*. Among many different EOs tested for their antifungal activities toward human dermatophytes, the EO of *Melaleuca alternifolia*, also called tea tree oil or melaleuca oil, seems to be more attractive to researchers in recent years (*90-94*). Tea tree oil contains about 100 components, which are largely monoterpenes, sesquiterpenes and related alcohols (*95*). An *in vitro* study to determine the MIC of tea tree oil against *Trichophyton rubrum* found it to be 0.1% (v/v) (*92*). In controlled clinical trials, tea tree oil cream was more effective in reducing symptoms than eliminating the infections (*91*). Combining tea tree and lavender (*Lavandula angustifolia*) EOs together showed synergistic activity against the dermatophyte infection (*93*). EOs from other plants have also shown promising antifungal activities against human dermatophytes (*96-98*).

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Mechanisms of Antifungal Action

EOs, as many other phytochemicals, are for the defense of plants themselves against different invading organisms including fungi, bacteria and viruses. The mechanisms involved in the antibacterial, antifungal and antiviral activities may have been similar. Like the antibacterial EOs, monoterpenoids and phenylpropanoids play important roles in the antifungal activity. The most active compounds, such as eugenol, thymol and carvacrol, are phenolic in nature, implying that the antifungal mechanisms of these EOs are generally similar to phenolic compounds, which interfere with function of the cytoplasmic membrane and proton transport (*99*). Conner and Beauchat (*100*) studied antifungal activities of EOs against food spoilage yeasts and indicated that EOs may inhibit enzyme systems, including those involved in energy production and synthesis of structural components. The research group has demonstrated energy depletion in yeasts caused by different EOs including clove, thyme, oregano and cinnamon (*101*).

To understand the effects of EOs on fungal cells, Svircev *et al.* (*102*) observed ultrastructure of the fungal conidia, intra- and intercellular components of *Monilinia fructicola* fumigated with thymol vapor using scanning electron microscopy (SEM). The electron micrographs clearly exhibited that thymol crystals attached to the surface of the fungal cell wall. Thymol vapors affected almost all fine structures of fungi in samples collected from the surface of the plum fruit. Sections of germ tubes, appressoria and surface hyphae exposed to thymol vapors were characterized by disrupted and disorganized cytoplasmic organelles (*102*). Similarly, using light and SEM, Soylu *et al.* reported that in *P. infestans* hyphae exposed to both volatile and contact phase EOs, considerable morphological alterations were found, including cytoplasmic coagulation, vacuolations, hyphal shrivelling and protoplast leakage (*72*).

Antiviral Activity of EOs

Compared to the studies conducted against bacteria and fungi, the volume of literature related to the antiviral activity of EOs is scarce. Most of the studies on the antiviral activity of EOs have been focused on herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), the two common human pathogens that cause epidermal lesions in and around the oral cavity, the eye, in the pharynx, and the oesophagus as well as in the mucous membrane of the genitals (*103*). Infections in immunocompromised patients are usually more severe than in immunocompetent hosts. The incidence and severity of disease produced by herpes simplex viruses have been increasing in recent years (*104*), especially in the immunocompromised hosts where viral resistance to acyclovir, a synthetic antiviral drug, represents a particular problem (*105*). This trend has led to a search for alternative antiherpetic agents that have a wide range of efficacy without serious adverse effects, and which are effective for viral strains resistant to current antiviral agents.

The EO of *Salvia fructicosa*, containing mainly 1, 8-cineole had strong virucidal activity against herpes simplex virus 1 (*106*). When the virus was exposed for 30 min to various concentrations of the EO of *Salvia fructicosa* or its main components, 80% of the virus was inactivated by an EO concentration of 0.2%, while at higher concentration (0.4%), this effect is enhanced by almost 4 $\log_{10}$ values (*106*). All of the main components showed high virucidal action, but thujone was the most active. Pure thujone at a concentration of 0.1% inactivates 95% of infectious virus particles, while the percentage of virus inactivation by 1,8-cineole or camphor is low (35% and 0%, respectively). The virucidal action of thujone is accelerated by almost 5 $\log_{10}$ values at higher concentrations (0.2%) (*106*).

Summary

Plant EOs are a group of highly versatile bioactive compounds. Monoterpenoids such as geraniol and carvacrol and phenylpropanoids such as eugenol and cinnamaldehyde are the major, and perhaps the most important, components of the various essential oils. These natural products, due to their

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lower toxicity to humans, are a good source of antibacterial agents, particularly against food-borne pathogenic bacteria such as *E. coli*, and *Salmonella typhimurium*. EOs or their major components can therefore potentially play an important role in food safety, preventing food from contaminations before, during and after harvesting and processing. Similarly, these small molecules may also be used to control fungi that cause food decay, thus extending the shelf life of fresh produce. EOs can also play an important role in food safety by inhibiting the growth and mycotoxins production of important food-, feed- and soil-borne fungi. The antiviral and antifungal activity of certain essential oils and their components against human diseases provide a safe alternative to synthetic drugs, particularly in immunocompromised individuals.

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Chapter 19

Antibacterial Actions of Ginkgolic Acids and Related Mechanisms

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Ginkgolic acids (GAs) are alkylphenols found in the *Ginkgo biloba L.* Although they have some undesirable actions to human body and have been limited in the extract preparation of Ginkgo leaves, they have wide bioactivities, such as antibacterial and antifungal actions, effects as molluscicide, inhibition on some key enzymes for the metabolism in pests, uncoupling oxidative phosphorylation of mitochondria, etc. The activities of GAs against Gram-positive bacteria, especially against the Methicillin resistant *Staphylococcus aureus* (MRSA) and their structure-activity relationships are presented in this chapter. The related mechanism is also explored.

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Background

Ginkgolic acids (GAs) are alkylphenols found in the *Ginkgo biloba L,* the sole survivor of the botanical family Ginkgoaceae. With a history of over 200 million years, Ginkgo is considered the oldest plant as a "living fossil" to survive on earth (*1*). The extracts from *Ginkgo biloba* leaves (GBE) is the most widely used phytopharmaceutical clinically for the improvement of blood circulation and treatment of dementia (*2, 3*). EGb761 is the first standardized preparation patented by Schwabe (*4*) in German for medical use. This pharmaceutical preparation (EGb761 formula) fulfilled the requirements in the Monograph of the Commission E of the former Federal Health Authority (Bundesgesundheitsamt, BGA), which has now been partly taken over by the European Scientific Cooperative on Phytotherapeutics (ESCOP) (*5*). Except the ginkgolides (terpene trilactones) and ginkgo-flavonoids (flavonols and their glycosides) recognized as the main active ingredients in the GBE, GAs have been treated as hazardous compounds with suspected cytotoxic and allergenic properties. So the content of GAs should not exceed 5 ppm in the standardized preparation by the above requirement. In recent years, many studied unveiled that GAs have wide bioactivities, which may endow Ginkgo some remarkable properties and genetic tenacity surviving over millions of years, e.g. resistant to diseases and pests. The research on the GAs' bioactivities would not only explore their position in their defense system, but makes us fully use this precious resource from the nature as well. This chapter is focused on their antibacterial actions.

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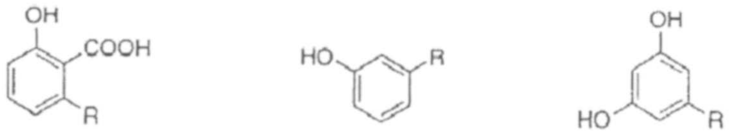
1. Structures of Ginkgolic Acids and Related Compounds

GAs belongs to alkylphenols, named as 6-alk(en)ylsalcylic acids, which are also known as anacardic acids (from cashew nut shell oil) . There are three different classes of alkylphenols (ginkgolic acids, ginkgols and bilobols) found in various parts of *Ginkgo biloba,* mainly in Ginkgo exocarps. Only the first two mentioned classes have been detected in Ginkgo leaves (*6*).

Structures and analogues

The structures for three classes of alkylphenols (ginkgolic acids, ginkgols and bilobols) found in *Ginkgo* are shown in Figure 1.

Synonyms for ginkgolic acids are 6-alkylsalicylic acids, 2-hydroxy-6-alkylbenzoic acids or anacardic acids, and synonyms for ginkgols are 3-alkylphenols or cardanols, while synonyms for cardols are bilobols, 5-pentadec-8[*Z*]-enylresorcinol. The alkyl side chain varies from 13 to 17 carbons in length with



Ginkgolic acids	Ginkgols	Cardols
R = $C_{13}H_{27}$ (C13:0)	R = $C_{13}H_{27}$ (C13:0)	R = $C_{15}H_{31}$ (C15:0)
R = $C_{15}H_{31}$ (C15:0)	R = $C_{15}H_{31}$ (C15:0)	
R = $C_{15}H_{29}$ (C15:1)	R = $C_{15}H_{29}$ (C15:1)	
R = $C_{17}H_{33}$ (C17:1)	R = $C_{17}H_{33}$ (C17:1)	
R = $C_{17}H_{31}$ (C17:2)		

Figure 1. Structures for three classes of alkylphenols in Ginkgo (6).

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zero to two double bonds, expressed in C15:0 or C17:2, etc. The double bonds possess the *Z*-configuration. When only one double bond is present, the position is most frequently in carbon 8 (*6*).

Separation and purification

GAs and related alkylphenols constitute major components of the lipid fraction during extracting of Ginkgo exocarps. As these compounds are also present in crude leaf extracts, the removal of GAs from the extract in the production process guarantees therapeutical use, requesting a maximal concentration of 5 ppm GAs in the preparation.

A chromatographic procedure for the preparative isolation of GAs from Ginkgo biloba leaves was developed, which consisted of a combination of normal-phase, reversed-phase and argentation chromatography. The GAs was characterized by means of UV, ^{1}H-NMR and ^{13}C-NMR spectroscopy, and mass spectrometry after silylation. A 15 cm C18 RP-HPLC column connected in series with a 20 cm silver(I) loaded cation exchanger HPLC column in combination with the solvent methanol-water (93:7) acidified with 0.1% formic acid was capable of separating the GAs (C13:0, C15:1, C17:2, C15:0 and C17:1) within 21 min on an analytical scale. Detection took place by UV at 311 nm. The separation is a good starting point for the development of a quantitative procedure for the five major GAs in Ginkgo leaves and standardized extracts (*7,8*). The extraction of GAs from the Ginkgo exocarps and leave for industrial application can be fulfilled by some organic solvents, e.g. hexane, petrol ether, etc. GAs was selectively isolated as calcium ginkgolates. The acid-free ginkgo exocarps was treated with liquor ammonia and extracted with hexane/ethyl acetate (98:2) to separate the mono phenolic component, cardanol. Subsequently, ammonia solution was extracted with ethyl acetate/hexane (80:20) to obtain cardol (*9*).

The separation of GAs can also be achieved by the technology of supercritical CO_2 fluid extraction. The optimum conditions for supercritical CO_2 fluid extraction was 30 MPa, 45^0C, extraction time 6 h and the flow rate of CO_2 2L/min. Then the separated GAs was determined by assay of HPLC. This technology of supercritical CO_2 fluid extraction excelled the traditional solvent extraction in high yield, high purification and easy operation (*10*).

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Analysis

The common sensitive and selective method for qualitative analysis of GAs in the leaves and exocarps of Ginkgo biloba is based on liquid chromatography-electrospray mass spectrometry (LC-ES-MS). In its negative ion mode, calibration curves with good linearity were obtained in the range of 0.1-10 ug/g. Five kinds of GAs (C13:0, C15:0, C15:1, C17:1 and C17:2) can be determined. The relative percentage content of GA-C13:0, C15:1 and C17:1 was above 94%. The optimized method was also applied to verify whether the amount of ginkgolic acids was below 5 ug/g in a standardized leaf extract preparation. The method is very specific for the analysis of GAs with no interferences from the sample matrix (*11,12,13,14*).

Due to a number of GAs with different side chains exist in Ginkgo leaves and exocarps, this makes their analysis by conventional chromatographic methods more complex. So the ^{1}H-NMR spectrometry was developed to the analysis of the total content of ginkgolic acids in leaves of Ginkgo biloba and the extract preparation in the absence of chromatographic purification. For this analysis, protons H-3, H-4, and H-5, which are well separated in the range 8 (ppm) 6.5-7.5 in the ^{1}H-NMR spectrum, were utilized. For further confirmation, the correlations of H-3, H-4 and H-5 were examined by ^{1}H-^{1}H COSY spectra in all extracts. The quantity of the compounds was calculated from the relative ratio of the integral of each peak to the integral of the peaks of a known amount (100 ug) of anthracene used as an internal standard. The quantitative results obtained by ^{1}H-NMR analysis were compared with those obtained by GC, which showed that the ^{1}H-NMR method allows a simple quantification of total ginkgolic acids in Ginkgo extracts without any pre-purification steps (*15*).

A competitive enzyme-linked immunosorbent assay (ELISA) for ginkgolic acids (GAs) was developed using monoclonal antibody (MAb) 9F raised against 6-(13-formylheptyl) salicylic acid covalently coupled to bovine serum albumin (BSA). ELISA, at an effective measuring range of 300 ng/ml~1 ug/ml of GA15:1, was successful in detecting GAs content in ginkgo leaves and standardized extracts due to the lack of cross-reactivity against various related compounds. The sensitive and simple immunoassay developed was validated to be specific for the quantitative determination of total GAs content in ginkgo crude drugs with no interference from the sample matrix (*16*).

Synthesis

It was found that GAs can be efficiently synthesized in seeds of Ginkgo biloba from immature. The seeds were incubated with ^{14}C-labeled acetic, malonic and palmitoleic acids, glucose, and other potential precursors. Levels of ^{14}C in common lipids and in GAs, and the distribution of ^{14}C in GAs were determined. The results show that the salicylic moiety is synthesized by a polyketide pathway via malonic acid. The chain moiety for GAs synthesis is in a different state of activation and/or site than chains that are used for synthesis of the common lipids. Labeled shikimic acid did not contribute ^{14}C to GAs, nor to other lipids, and palmitoleic acid was incorporated only into common lipids (*17*).

For the analysis of the structure-activity relationship, some GA analogues can be synthesized based on the structure of salicylic acid (*18, 41, 48*).

2. Position of Ginkgolic Acids in Ginkgo and Ginkgo Leave Extract Preparation

Extract of *Ginkgo biloba L* leaves

Ginkgo biloba is among the most sold medicinal plants of this world with estimates of worldwide annual sales varying from a conservative US M$ 450 to over 1 billion US $ in 1998. Most of the sales concern special extracts from the leaves which have been standardized for their content of terpene trilactones and flavonol glycosides.

There are over 120 published clinical studies on ginkgo, primarily from Europe. Four major concepts of actions for the extract of Ginkgo biloba obtained from the experiments in vivo and vitro, and the observation of clinical trials are as follows (*2*): (1) the vasoregulatory action of protecting blood vessels and various tissues , which explains its effects in regulating cerebral insufficiency status, neurosensory disturbances and peripheral occlusive arterial diseases; (2) the cognition-enhancing action which relates to its use in treating Alzheimer's disease and various dementias; (3) the "stress-alleviating" action which is useful in explaining its "anxiolytic-like"/ "antidepressant-like" effects; (4) the gene-regulatory action which provides basis for explaining why most of the clinically beneficial effects of the extract require repeated administration.

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Unfortunately the leaves also contain considerable amount of undesirable ingredients i.e. ginkgolic acids, for human body which should be removed during processing. In crude Ginkgo extracts, ginkgolic acids (GA) and related alkylphenols have been recognized as hazardous compounds with suspected cytotoxic and allergenic properties.

The cytotoxic potential of GAs was confirmed on the human keratinocyte cell line HaCaT and the rhesus monkey kidney tubular epithelial cell line LLC-MK. The morphological evaluation of LLC-MK_cells indicated that the cytotoxic activity of GA in these cells was primarily mediated by transformation of mitochondria, which is probably induced by uncoupling of oxidative phosphorylation (*19*).

The study showed that GA-induced death of cultured chick embryonic neurons was mediated by specifically increasing the activity of protein phosphatase type-2C (*20*). The embryotoxic effect of different fractions derived from Ginkgo containing GAs was checked in the hen's egg test (HET). A fraction enriched for ginkgolic acids (16%) and biflavones (6.7%) was found to induce death of 50% of the chick embryos (LD50) at a dose of 1.8 mg/egg (33 ppm) (*21*).

The immunotoxicologic effects were checked by the popliteal lymph node assay (PLNA) in the mouse causing lymphoproliferative reaction (LPR) in the ipsilateral popliteal lymph node. Although the LPR was observed for a crude aqueous-ethanolic extract from Ginkgo leaves containing GAs (*22*), further study of chromatographic separation revealed that the immunotoxicologic effect merely occurred after application of the subtraction which contained more than 70% biflavones (*23*).

Furthermore, some study proved that though the guinea pigs could be sensitized successfully with the pure GAs, the animals could not be sensitized with the leaf extract. Leaf extracts of Ginkgo biloba taken orally or given by infusion to treat diffuse cerebral disturbances can be considered safe, even when they might contain up to 1,000 ppm of the sensitizing ginkgolic acids (*24*).

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Role of GAs in the defense system of Ginkgo

Ginkgos have survived over millions of years because of their genetic tenacity. They are long-lived trees, remarkably resistant to diseases, pests, and fires. They are also extremely tolerant to air pollution, therefore they are often planted in major urban areas. Ginkgo was also famed for its miraculously surviving from the catastrophe of the atomic bomb in Hiroshima during the Second World War as “the indestructible tree”.

Undesirable ingredients in Ginkgo for human being do not mean the waste produced by the nature, but with definite usage for its own system waiting for discovery.

For a long time, the peasants in China and Japan knew that the parasite insects did not attack the gingko as often as other trees. They concluded that some elements we know as GAs today must be acting as a repellent, an active ingredient with insecticidal qualities. So they often used the decoction of ginkgo leaves as the insecticide for their crops.

Contents of GAs in Ginkgo leaves and exocarps

The contents of GAs in the dried Ginkgo leave isolated and determined by dual column HPLC were about 0.4% (*8*). The contents of GAs in the Ginkgo exocarp are much higher than that in leaves, which amount determined by Jaggy H were 3.54% (*7*), while the GAs contents detected by Yang Liu-Qin were 5.46% (*25*). The percentage of different GAs in Ginkgo leaves and exocarps are shown in Table1.

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3. The Bioactivities of Ginkgolic Acids

Many researches showed the wide bioactivities of GAs besides their antibacterial actions. The main bioactivities are summarized as following:

Antifungal activity

The antifungal activity of crude extract and GAs mixture from ginkgo exocarps were performed on twelve plant pathogenic fungi (*27*). The crude extract and GAs mixture produced good inhibition on nine fungi of these plant pathogenic fungi (shown in Table 2). There was no obvious inhibition on another three fungi, i.e. *Phytophthora capsici*, *Gibberella fujikuro*, *Botrytis cinerea.* The GA contents form the crude extract and the GA mixture were 58.6 % and 86.3 % respectively.

Zoosporicidal activities

Some GA analogues separates and purified from the unripe fruits of Ginkgo biloba showed motility inhibition followed by lysis of zoospores of the phytopathogenic *Aphanomyces cochlioides.* Among them, the GA-C22, a GA derivative showed potent motility inhibition (98% in 30 min) followed by lysis (55% in 3 h) of the zoospores at 1 x 10^{-7} M solution. A brief study on structure-activity relationships revealed that a carboxyl group on the aromatic ring and an unsaturated side chain in the GA derivative are important for strong motility inhibitory and lytic activities against the zoospore (*28*).

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Table 1. Percentage of different ginkgolic acids from Ginkgo leaves and exocarps. (%)

	C13:0	C15:0	C15:1	C17:1	C17:2
From dried leave(*8*)	5.86	4.67	37.0	49.16	3.28
From dried exocarps(*26*)	20.7	3.3	51.6	21.1	3.3

Table 2. Inhibition of crude extract and ginkgolic acid mixture from ginkgo exocarps on plant pathogenic fungi (*27*). (%)

Fungi	crude extract (ug/mL)			GA mixture (ug/mL)		
	400	200	100	400	200	100
Gibberella zeae	42.1	24.0	13.0	35.1	17.3	6.7
Sclerotinia allii	6.5	3.7	2.4	15.3	12.7	10.4
Rhizoctonia zeae	43.1	31.9	17.7	39.3	30.9	18.1
Colleto trichum nigrum	31.9	21.5	15.8	22.6	21.5	16.8
Fusarium oxysporum F.sp.niveum	13.6	11.8	6.4	9.5	7.5	3.5
Fulvia fulvum	37.1	33.3	27.7	39.2	36.2	28.6
Phoma arachidicola	55.0	50.3	35.2	57.0	48.6	40.2
Alternaria solani	37.5	23.5	17.8	42.2	38.6	26.0
Cercospora personata	42.4	28.5	11.6	44.9	27.9	18.9

Molluscicide activity

Schistosomiasis, one of the largest of human health problems in the tropics and some other place, is a parasitic disease mainly carried by snail vectors. Control of snail vectors by molluscicides can be a rapid and efficient means for reducing or eliminating the transmission of this disease. Molluscacides of plant origin are currently receiving considerable attention due to their relatively harmless biodegradative properties.

The molluscicide activity of GAs with C15 side chain against the South American freshwater snail *Biomphalaria glabratus* was proved and the study on their structure-activity relationship unveiled that the activity increases in direct proportion to the degree of unsaturation in the alkyl side chain. The LD50 for the GA-C15:3, C15:2, and C15:1, isolated from another source *Anacardium occidentale L.* (cashew) nut shell were 0.3 ppm, 0.6 ppm and 1.0 ppm respectively (*29*). When the hexane extract of the cashew nut shell by precipitation with cupric sulfate plus sodium hydroxide to obtain better stability for GAs, the GA (sodium hydroxide) presented the molluscicide activity at 4 ppm (*30*).

The effects of GAs extracted from the ginkgo exocarps on killing *Oncomelania hupensis,* the main intermediate for *Schistosoma japonicum* in China, were examined in our laboratory.

We found that ginkgolic acids have the mulloscicidal activities different from that of niclosamide-the only molluscacide recommended by WHO with the following two characteristics: GAs could effectively inhibit snail's climbing up to prevent its escape and GAs could produce mulloscicidal effects quickly, mainly within 24 hours. Based on the results of our experiments, the order of the molluscicidal activities for the 5 ginkgolic acid monomers was as following: GA(13 : 0) > GA(15 : 1) > GA(15 : 0) > GA(17 : 1) > GA(17 : 2). The LC_{50} (24 h) for GA(13 : 0), GA(15 : 1) and GA(15 : 0) were 13.17 mg/L, 18.57 mg/L, 26.33 mg/L, respectively. They occupy 70% content in the total extracted ginkgolic acids, composing the main molluscicidal activities for the latter, in which the LC_{50} (24 h) was 6.67 mg/L (*31,32*). Based on these results, a new molluscicide mainly formed by GAs is under research and development.

Inhibition on some key enzymes for the metabolism in pests

The GA-C22:1 with the omega 5-unsaturated chain, a GA congeners from *Anacardium occidentale L.* (cashew) nut shell, was found to be a effective inhibitor of both potato lipoxygenase and ovine prostaglandin endoperoxide synthase with approximate IC50 of 6 uM and 27 uM, respectively. If the side chain was saturated, it still has nearly the same inhibitory action toward these enzymes. However, its dimethyl derivative was a poor inhibitor of prostaglandin

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endoperoxide synthase and with moderate (32%) inhibition on lipoxygenase even at 135 uM (*33*).

GAs, 2-methylcardols, and cardols isolated from the cashew [Anacardium occidentale] (Anacardiaceae) fruit have been found to exhibit inhibitory activity on the tyrosinase, which is one of the key enzymes for the insects during ecdysis (*34*). It was also found that GA-C15:3 produced obvious inhibition on the generation of superoxide radicals induced by xanthine oxidase. The salicylic acid moiety and alkyl side chain in GAs are associated with the cooperative inhibition and hydrophobic binding, respectively (*35*).

The glycerol-3-phosphate dehydrogenase (EC 1.1.1.8, GPDH) is a key enzyme in the synthesis of triacylglycerol (TG) converting glycerol-3-phosphate to lyceraldehyde-3- phosphate in the presence of NADH. Therefore, GPDH inhibitors are interesting from the point of antiobesity. It was found GAs are good inhibitors of GPDH (*36*).

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Antitumor Activities

The inhibitory effect of GAs from ginkgo exocarps on human tumor cells and normal cells lines was examined by MTT assay. The results suggest that GAs obviously inhibited the growth of tumor cells in concentration of 5.0 ug/mL in vitro without affecting the normal cells (2*6*).

Histone acetyltransferases (HATs) are a group of enzymes that play a significant role in the regulation of gene expression. These enzymes covalently modify the N-terminal lysine residues of histones by the addition of acetyl groups from acetyl-CoA. Dysfunction of these enzymes is often associated with the manifestation of several diseases, ranging from neurodegenerative disorders to cancer. These enzymes thus are potential new targets for therapeutics. It was found that GAs was a potent inhibitor of p300 and p300/CBP-associated factor HATs activities. Although it does not affect DNA transcription, HAT-dependent transcription from a chromatin template was strongly inhibited by GAs. These compounds would be useful as biological switching molecules for probing into the role of p300 in transcriptional studies and may also be useful as new chemical entities for the development of anticancer drugs (*37,38*).

Uncoupling effect on oxidative phosphorylation of mitochondria

The uncoupling effect of GAs on oxidative phosphorylation was checked on rat liver mitochondria using succinate (plus rotenone) as a substrate. Four GAs with C15:0, C15:1, C15:2 or C15:3 as an alkyl side chain exhibited uncoupling effects similar to the classical uncoupler, 2,4-dinitrophenol on ADP/O ratio, state 4 and respiratory control ratio (RCR). The GA with C15:1 side chain was most effective for uncoupling of these compounds. Salicylic acid, which has no alkyl side chain, exhibited a very weak uncoupling effect on oxidative phosphorylation. When the carboxyl group was lost to the corresponding cardanols, uncoupling activity dramatically decreased. These results suggest that the C15 alkyl side chain as well as the carboxyl group may play an important role in promoting the uncoupling activity of GAs in mitochondria (*39*). The results provide the evidence for a unique function of GA, behaving as both an electrogenic (negative) charge carrier driven by membrane potential and a 'proton carrier' that dissipates the transmembrane proton gradient formed (*40*).

4. Activity of Ginkgolic Acids against Gram-Positive Bacteria and their Structure-Activity Relationships

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Antibacterial activity

The antibacterial activity of GAs was first found for the alkylphenols isolated from the *Anacardium occidentale* (Anacardiaceae) cashew nut shell oil (*41*). Many studies showed that GAs has much stronger inhibiting effects on Gram-positive (G+) bacteria (*42,43,44*) than on Gram-negative (G-) bacteria, except on the *Helicobacter pylori* the causative agent of acute gastritis (*45*). The study performed in our laboratory also showed that one of the GAs, GA-C13:0 produced inhibitory action against G+ bacteria, i.e. *Staphylocococus aureus* (ATCC 6538) and *Bacillus subtilis var. niger* (ATCC9372) with the MIC of 25 ug/ml among the five infectious organisms selected, containing two G-bacteria, *E.coli* (ATCC 8099) and *Pseudomonas aeruginosa,* one fungus, *Candida albicans* (ATCC 10231) and two above G+ bacteria (shown in Table 3) (*44*). The anti-microbial activity of GAs was enhanced with the increasing of its concentration and the acting time.

The GAs with C15 alkyl side chain exhibited the most effective inhibitory activity against the G+ bacteria tested with potent but narrow spectrum of antibacterial activity *(42)*, especially against G+ bacteria such as *Streplococcus mutans (Sm), Brevibacterium ammoniagenes (Ba), Staphylococcus aureus (Sa), Bacillus subtilis (Bs), and Propionibacterium acnes(Pac)*(shown in Table 4).

The salicylic acid with no alkyl side chain exhibited weak but broad antimicrobial activity against almost all of the microorganisms tested, and this property may make it be used as a cosmetic preservative.

The C15 alkyl side chain in GAs plays an important role in the antibacterial activity. The activities of C15 with 3 double bound in the alkyl side against *S. mutans* and *S. aureus* were 2048 and 64 times more effective than salicylic acid, respectively. The antibacterial activity against G+ bacteria was increased accom-

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Table 3. Inhibitory effects of GA-C13:0 on 5 infectious microorganism species.

infectious organism	MIC (ug/mL)	Control (cfu/mL)
E.coli (ATCC 8099)	>200	6.2×10^5
Pseudomonas aeruginosa	>200	5.8×10^5
S.aureus (ATCC 6538)	25	7.8×10^5
B. subtilis var. niger (ATCC 9372)	25	6.5×10^5
Candida albicans (ATCC 10231)	>200	5.0×10^5

Table 4. Activities of ginkgolic acids against several Gram-positive bacterial species (MIC, ug/mL) *(42)*

	GA-C15:3	GA-C15:2	GA-C15:1	GA-C15:0	SA
Bs	3.13	6.25	6.25	100	400
Ba	3.13	6.25	6.25	50	400
Sa	6.25	25	100	>800	400
Sm	1.56	3.13	3.13	>800	3200
Pac	0.78	0.78	0.78	0.78	400

Bs: Bacillus subtilis ATCC 9372, *Ba: Brevibacterium ammoniagenes* ATCC 6872
Sa: Staphylococcus aureus ATCC 12598, *Sm: Streptococcus mutans* ATCC 25175
Pac: Propionibacterium acnes ATCC10145

panying the increase of double bonds in the C15 side chain. The GAs with C15:1~3 were also isolated from the chasew *A. occidentale* apple, which has been consumed by many people. In the case against *P. acnes (Pac)*, all of the four GAs showed very strong inhibition on the growth of this bacterium with the MIC of 0.78 ug/mL. And the double bonds inside of the C15 side chain makes their activities against *S. mutans* and *S. aureus* dramatically increased compared with the GAs without double bond in C15. This action can also be seen for their activities against *B. subtilis and B. ammoniagenes (42).* So GA-C15:0 can be used as a standard for its simple structure and stable property to check the antibacterial activity of GA congeners, and more importantly, the synthesis of its various congeners can easily be accomplished.

In view of the increasing importance of controlling specific bacteria such as *S. aureus, P. acnes, and S.mutans,* the GAs may be used for skin and dental caries caused by these bacteria *(42).*

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Structure-activity relationship

The study on the structure-activity relationship of GAs showed that the C15 alkyl side chain plays an important role in increasing the antibacterial activity. The unsaturation in the alkyl side chain is not essential in eliciting activity but is associated with increasing the activity.

Table 5 shows the activities against G+ bacteria by a series of synthetic GA analogues with different carbon atoms in the side chain. As a result, their structure-activity relationships can be summarized *(42)*. Compared with the GA-C15:0, the activities against G+ bacteria were increased when the numbers of carbon atom increased, the GA-C12:0 becoming the most effective one even stronger than C15:3 for the *P. acnes and B. ammoniagenes.* And their antibacterial activities were completely lost when the carbon atoms reached twenty. The C17:1 in GA also did not show any antimicrobial activity up to 800 ug/mL. Besides the antibacterial activity, the GA-C8:0 also exhibited noticeable antifungal activity against a mold, *Peniciilium chrysogenum*, with a MIC of 12.5 ug/mL.

5. The activities of ginkgolic acids against MRSA and related mechanism

Methicillin resistant *Staphylococcus aureus* (MRSA) represents a therapeutic problem of increasing importance, especially in immune-compromised hospital patients. Because of its mutatious, *S. aureus* can easily develop resistance to commonly used antibiotics. The GA-C15:3 was reported to be an excellent anti-

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Table 5. Activities of ginkgolic acid's analogues against several G+ bacterial species. (MIC, ug/mL) *(42)*

	C6:0	C8:0	C10:0	C12:0	C15:0	C20:0
Bs	50	12.5	3.13	3.13	100	>800
Ba	200	25	3.13	0.78	50	>800
Sa	100	12.5	3.13	6.25	>800	>800
Sm	200	50	3.13	1.56	>800	>800
Pac	100	3.13	1.56	0.39	0.78	>800

Bs: Bacillus subtilis ATCC 9372, *Ba: Brevibacterium ammoniagenes* ATCC 6872
Sa: Staphylococcus aureus ATCC 12598, *Sm: Streptococcus mutans* ATCC 25175
Pac: Propionibacterium acnes ATCC10145

Table 6. Antibacterial activity of ginkgolic acid alone and in combination with methicillin against MRSA. (MIC, ug/mL) *(49)*

		C15:3	C15:2	C15:1	C15:0
alone		6.25	12.5	100	1600
combination	GA	1.56	1.56	3.13	50
	Methicillin*	25	50	25	25
FIC index		0.281	0.187	0.063	0.063

*The MIC of methicillin alone was 800 ug/mL.

MRSA agent. It can rapidly kill MRSA without any viable colony-forming units detected after 6 h in concentration of 6.25 mg/mL (*46*).

Synergistic effects of GAs and methicillin against MRSA

The synergistic effects of GAs in combination with methicillin against *Staphylococcus aureus* ATCC 33591 (MRSA) was fully investigated in Dr.Isao Kubo's laboratory.

Among these four GAs with C15 alkyl side chain, GA-C15:3 exhibited the most potent activity against this MRSA strain with an MIC of 6.25 mg/mL whereas GA-C15:0 did not show any activity up to 1600 mg/mL (shown in Table 6) with the similar results against G+ bacteria mentioned above.

A synergistic effect was observed in the combination of C15:3-anacardic acid and methicillin, and the minimal inhibitory concentration (MIC) of methicillin was decreased from 800 to 1.56 mg/mL for MRSA ATCC 33591 by combining with 3.13 mg/mL (equivalent to 1/2 MIC) of C15:3-anacardic acid.

The synergistic effects were expressed as the fractional inhibitory concentration (FIC) index (*47*). In this method, synergism is defined as an FIC index of <0.5, additivity as an FIC index of 0.5-1.0, and antagonism as an FIC index of >1.0.

There was a positive correlation between the FIC indices and the number of double bonds in the alkyl chain of GAs. The greatest synergism was observed in the combination of methicillin and GA-C15:0 with the FIC index of 0.281, and the least synergism was observed in the combination with GA-C15:3. While the MIC of GA-C15:1 was 100 mg/mL and that of methicillin was 800 mg/mL against MRSA alone, the equal effect was achieved by 3.13 mg/mL of GA-C15:1 in combination with 25 mg/mL of methicillin. In other words, the MIC of methicillin was lowered from 800 to 25 mg/mL in combination with 3.13 mg/mL (equivalent to 1/32 MIC) of GA-C15:1. The activity of methicillin was enhanced 32-fold by combining with GA-C15:1. Although the GA-C15:0 alone did not exhibit any activity up to 1600 mg/mL, it can effectively inhibited the growth of this bacterium in concentration of 50 mg/mL when combination with 25 mg/mL of methicillin (*48, 49*).

Structure-activity relationship

The synthetic GA analogues with different length of the alkyl chain were tested against the same MRSA strain for analysis of their structure-activity relationship. The results are shown in Table 7.

The activity of GA analogues against MRSA was significantly affected by the lengths of the alkyl chain. Their antibacterial activity became greater as the length of the alkyl chain increased, and the maximum activity was observed in GA-C10:0 and GA-C12:0 with the MIC of 6.25 mg/mL. The activity drastically

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Table 7. Antibacterial activity of ginkgolic acid's analogues alone and in combination with methicillin against MRSA. (MIC, ug/mL) *(49)*

		SA#	C1	C5:0	C8:0	C10:0	C12:0	C20:0
alone		400	400	100	12.5	6.25	6.25	1600
combination	GA	200	200	50	3.13	1.56	1.56	---
	Methicillin	200	100	50	200	200	25	---
FIC index		0.750	0.625	0.563	0.500	0.500	0.281	---

#: SA: salicylic acid, *The MIC of methicillin alone was 800 ug/mL.

Table 8. Antibacterial activities of some ginkgolic acid isosteres (*51*).

R	Mol. formula	IC50(TCM)(uM)		MIC (ug/mL)			
		KinA/SpoOF	NR_{II}/NR_I	*S.aureus*	*MRSA*	*E.faecalis*	*E.faecium*
-O-C11	$C_{18}H_{28}O_4$	25	44	8	16	16	16
-O-C12	$C_{19}H_{30}O_4$	4.4	17	8	8	8	8
-O-C14	$C_{21}H_{34}O_4$	2.2	5.0	>64	>32	4	4
-O-C16	$C_{23}H_{38}O_4$	3.0	1.5	>128	128	8	8

dropped for the GA-C15:0 and the GA-C20:0 had no activity up to 1600 mg/mL. The results obtained indicate that the double bond in the alkyl side chain is not essential in eliciting the activity but is involved with increasing the activity (*48, 49*).

Overall, the synergistic effects of GA-C15 decreased with increasing the number of double bonds in the alkyl chain (C15:0>C15:1>C15:2>C15:3), while their antibacterial activity increased with increasing the number of the double bonds in the alkyl chain (C15:3>C15:2>C15:1>C15:0). This action pattern can be explained by the knowledge that the introduction of unsaturation or branching into the hydrophobic group increases the solubility of the surfactant in water.

Among the compounds tested, GA-C15:3, GA-C12:0, and GA-C10:0 were the most potent, each with an MBC of 6.25 ug/mL against MRSA. No differences in their MICs and MBCs were noted, suggesting that their activity is bactericidal.

The antibacterial activity of GA-C15:3 and GA-C12:0, penicillin G, and methicillin were tested against the six selected *S. aureus* strains for comparison. Both GA-C15:3 and GA-C12:0 were effective against all of the strains of *S. aureus* tested, with the MICs ranging from 1.56 to 12.5 ug/mL. The activity of GA-C12:0 is slightly more potent than that of GA-C15:3 against some strains tested.

The FIC index for GA-C15:3 in combination with methicillin was 0.281. The equally potent synergistic effect on antibacterial action was observed in combination with GA-C12:0, indicating that the double bond is not essential in eliciting the synergistic activity. The FIC index for this combination was also calculated as 0.281 (*49*).

Related mechanism

Some study showed that the bactericidal activity of GA-C15:3 against *S. aureus* was associated with the disruption of the membrane. The GA-C15 and their selected analogues were tested for their effects on the bacterial respiratory system. The GA-C15:3 inhibited the oxygen consumption of *M. luteus* ATCC 4698 and *P. aeruginosa* IFO 3080 cells when suspended with these bacterial cells (*50*). Furthermore, all GA-C15 analogues can inhibit the oxidation of NADH by a membrane fraction prepared from *M. luteus* cells, indicating that GAs can inhibit the respiratory chain enzyme activity. Interestingly, salicylic acid did not exhibit this respiratory inhibition, indicating that the C15-alkyl side chain is important to elicit this inhibitory activity. However, GA-C12:0 did not inhibit the bacterial NADH oxidase up to 30 ug/mL. The results obtained are consistent with the fact that GAs exhibit uncoupling effects on oxidative phosphorylation of rat liver mitochondria (*38*). Apparently, the number of double bonds in the side chain is not directly associated with the respiratory inhibition activity, but the alkyl side chain length appears to be related to the activity. It is worth noting that

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GA-C15 also inhibited NADH oxidase in bovine liver mitochondria, although to a lesser extent.

Many studies prove that the bacterial two-component regulatory systems (TCS) consisting of a histidine kinase sensory protein (HPK) and a response regulator protein (RR) are involved in the regulation of chemotaxis, porin expression, nitrogen metabolism and expression of virulence and resistance factors that are vital for survival inside the host organism. In response to an external stimulus, HPK utilizes ATP to autophosphorylate at a specific histidine residue; the phosphoryl group from the HPK-P, in turn, is transferred to an aspartyl residue within the conserved domain of RR, culminating in gene transcription. Sequence alignment shows homology as high as 30% among several RR within the same cell, especially in those domains involved in the phosphotransfer function. This suggests it may be possible to target multiple bacterial TCS within the same cell, making chromosomally determined drug resistance highly unlikely. Because TCS have not been detected in mammalian cells, a bacterial TCS inhibitor could be an ideal therapeutic agent with good selectivity against virulent bacterial strains such as MRSA, etc. One study showed that some GA isosteres can produce strong inhibition on the TCS (*51*).

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6. Some GA Isosteres with Antibacterial Activities

Based on the antibacterial activity and analysis of the structure-action relationship, a series of 6-oxa isosteres of GAs was synthesized. Their antibacterial activities were determined on some strains of bacteria, i.e. *S. aureus, MRSA, E. faecalis and E. faecium.*

The mechanism of their activity was also tested by the bacterial two-component regulatory systems, KinA/SpoOF and NR_{II}/NR_{I}. The alkyl side chain was replaced by an oxygen atom adding with a different length of alkyl chain. The isosteres with O-C14 and O-C16 were found to be among the most potent inhibitors of the bacterial two-component regulatory systems, KinA/SpoOF and NR_{II}/NR_{I}, (shown in Table 8). The isostere with O-C11 produced strong inhibition not only on the KinA/SpoOF and $NR_{II}/NR_{I,}$ but on all of the tested bacteria as well (*51*).

Summary

Investigation of the bioactivities of GAs, especially their antibacterial activity and the structure-activity relationship would not only unveil their position in the defense system of Ginkgo, but bring benefit to human being against some infectious bacteria and pests as well. As these compounds can also be extracted from the edible cashew apple and with biodegradative properties, they may be regarded as a new kind of biocide.

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Chapter 20

Antimicrobial Properties of Boron Derivatives

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This chapter reviews new and previously published data related to *in vitro* antimicrobial properties of boron derivatives, with an emphasis on susceptibility studies against bacteria, yeast, and filamentous fungi. Fundamental borate chemistry is discussed with respect to properties in biological systems. New facts providing insight into biostatic and biocidal mechanisms of action of boron compounds are presented along with critical factors that influence their antimicrobial performance.

Introduction

Although boron is naturally present and widely distributed in the environment, it generally occurs at relatively low concentrations. With the exception of a few rare boron fluoride minerals, boron is always found chemically bound to oxygen. Representing only about 0.001% of the Earth's crust, boron is omnipresent in rocks and soils and also occurs at measurable levels in virtually all natural waters. Ocean waters contain an average of 4.6 ppm; freshwater lakes and rivers usually have lower concentrations, typically around 0.1 ppm, but can contain significantly greater concentrations in areas of higher boron geologies (*1*). Most boron in the environment is present as naturally occurring boric acid. However, other borate compounds readily convert to and interchange with boric acid.

Boric acid has been known as a mild antiseptic for about a century. It was introduced by Godlee in 1873 as a companion drug to carbolic acid (*2*). Since its first introduction, boric acid has been widely accepted in clinical use for topical applications. As a fungistatic compound, it has been used successfully for treatment of yeast infections in obstetrics and gynecology (vulvovaginal candidiasis), especially with non-*Candida albicans* infections (*3*, *4*). Because of its nonspecific activity, boric acid is one of the most widely used topical agents for the management of otomycosis ear infections (*5*, *6*). Boric acid has also been used for 20 years to preserve urine samples while in transit to bacteriological examination (*7*).

While boric acid has been employed since the pre-bacteriological era, the introduction of newer antibacterial and antifungal agents, including antibiotics, sulfonamides, quaternary compounds, and others, has left boric acid with little scientific attention to its action as a microbiocide. As a result of this situation, there is a limited number of accurate laboratory studies evaluating *in vitro* antimicrobial activity of boric acid.

The objective of this chapter is to examine new and previously published data related to *in vitro* antimicrobial properties of boron derivatives, with an emphasis on susceptibility studies against bacteria, yeast, and filamentous fungi. The reader is presented with facts providing insight into biostatic and biocidal mechanisms of action of boron compounds along with critical factors that influence their antimicrobial performance.

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Fundamental Chemistry of Borates

Aqueous Chemistry

Boric acid is a weak Lewis acid. The slight acidity of boric acid solutions does not result from a tendency to donate protons, but rather from the ability to accept hydroxyl ions. Boric acid reacts with water, as given by Equation 1,

$$B(OH)_3 + 2\,H_2O = B(OH)_4^- + H_3O^+ \qquad \text{Eq. 1}$$

to give an equilibrium concentration of the $B(OH)_4^-$ anion. The equilibrium constant for this reaction ($K_a = 5.80 \times 10^{10}$ mol·L^{-1} at 25°C) is small enough that the proportion of boric acid in aqueous solutions at near-neutral pH is >99%. The relative concentration of the $B(OH)_4^-$ anion increases with increasing pH and becomes the dominant species above a pH of approximately 9.2. The monomeric $B(OH)_3$ and its conjugate base $B(OH)_4^-$ are the only significant boron species in a borate dilute solution (less than about 0.1 mol·L^{-1}).

Boric acid is moderately soluble in water and exhibits endothermic dissolution. The water solubility of boric acid therefore increases greatly with increasing temperature. The weak acidity of boric acid is due to its Lewis acidity, or tendency to accept electron donors, such as base the OH^-. Brønsted acidity, the tendency to donate protons, plays little role in the aqueous chemistry of boric acid. In a dilute aqueous solution, boric acid exists almost exclusively as monomeric $B(OH)_3$ and $B(OH)_4^-$. With an acid equilibrium constant approximately three million times lower than acetic acid, boric acid is a relatively weak acid compared to other common acids. A 1% aqueous solution of boric acid is only slightly acidic, exhibiting a pH of about 5.

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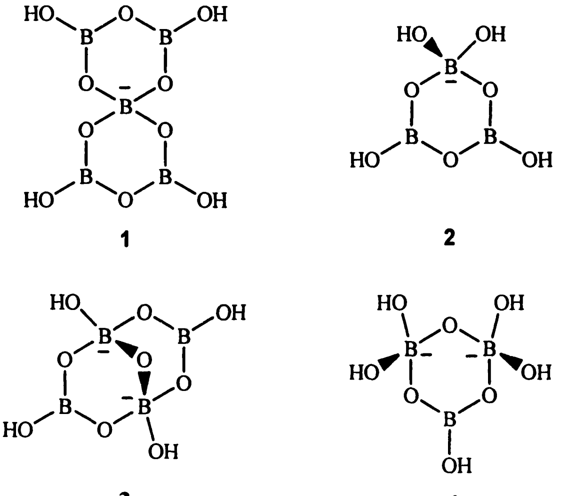
Although $B(OH)_3$ and $B(OH)_4^-$ are monomeric in dilute solutions, at concentrations above about 0.1 $mol{\cdot}L^{-1}$, condensed borate species form complexes that are often referred to as polyborates. Formation of these species is a result of the tendency of boron to form complexes with electron donor species, including oxygen, attached to boron itself. The most important polyborate species in aqueous solutions are the pentaborate anion, $B_5O_6(OH)_4^-$ (**1**), the triborate monoanion, $B_3O_3(OH)_4^-$ (**2**), the tetraborate anion, $B_4O_5(OH)_4^{2-}$ (**3**), and the triborate dianion, $B_3O_3(OH)_5^{2-}$ (**4**), shown in Figure 1. The population distributions of these species as a function of pH are shown in Figure 2 (*8*).



1 **2** **3** **4**

Figure 1. Polyborate anions occurring in relatively concentrated aqueous solutions: $B_5O_6(OH)_4^-$ *(1),* $B_3O_3(OH)_4^-$ *(2),* $B_4O_5(OH)_4^{2-}$ *(3),* $B_3O_3(OH)_5^{2-}$ *(4).*

The fact that polyborates only occur in concentrated solutions is biologically relevant, because boron is naturally present in both natural waters and biological systems only in low concentrations. Under these conditions, $B(OH)_3$ and $B(OH)_4^-$ are the only relevant boron species; in near-neutral pH, the concentration of the $B(OH)_4^-$ anion is minor. Therefore, boron in the soil, sea, freshwater lakes and streams, as well as plants and animals, is present mainly as boric acid, even if the source of boron is a borate salt.

Perborates

Perborates, or peroxoborates, are of particular relevance to biocidal applications. Their formulas are usually written as if the perborate anion was

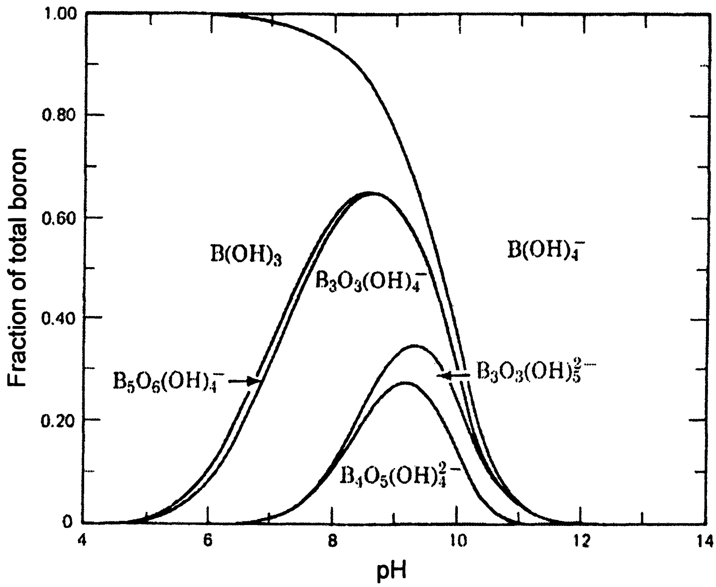
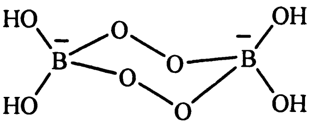


Figure 2. Estimated population distributions of borate species in aqueous solutions as a function of pH at 25°C and 0.4 mol·L^{-1} boric acid equivalent concentration. After Ingri (8).

BO$_3^-$. Thus, the three well-known sodium perborates are commonly written as NaBO$_3$·nH$_2$O (n = 1, 3, and 4) and referred to as the mono-, tri-, and tetrahydrates. However, these compounds contain the cyclic dianion **5**. Therefore, the more structurally correct formula is Na$_2$[B$_2$O$_4$(OH)$_4$]·nH$_2$O (n = 0, 4, and 6), and these compounds are more correctly referred to as anhydrous, tetrahydrate, and hexahydrate.



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The so-called mono- and tetrahydrates of sodium perborate, referred to as PBS-1 and PBS-4, respectively, are important commercial products. This nomenclature is based on the NaBO$_3$·nH$_2$O formula. Both have been used extensively as bleaching agents in home laundry detergent formulations. They

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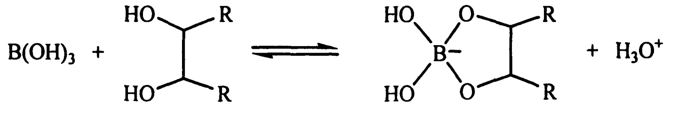
are white crystalline powders that decompose when dissolved in water to form peroxoborate species, including $(HO)_2BOOH$, $(HO)_3BOOH^-$, and $(HO)_2B(OOH)_2^-$, which ultimately decompose with the liberation of hydrogen peroxide. Thus, they provide a convenient means of handling latent hydrogen peroxide in a dry powder form. Tetrahydrate is manufactured in large quantities by the reaction of hydrogen peroxide with a sodium metaborate solution prepared from sodium hydroxide and sodium tetraborate pentahydrate. The monohydrate is produced by the dehydration of the tetrahydrate. The tetrahydrate contains 10.4 wt% of active oxygen. Its decomposition in water is accelerated by metal catalysts and increasing temperature. Without an added stabilizer, solutions will slowly lose active oxygen at room temperature. Water solubilities of the tetrahydrate are 2.5 wt% at 20°C and 3.6 wt% at 29°C. Sodium perborate monohydrate, $NaBO_3{\cdot}H_2O$, has a higher active oxygen content of 16.0 wt% and also exhibits a faster rate of dissolution in water than tetrahydrate.

Borate Esters

Boric acid forms esters with alcohols, in the simplest case, according to Equation 2. These esters are generally unstable under hydrolysis and decompose rapidly to boric acid and alcohol when exposed to water. However, reactions of boric acid with 1,2- and 1,3-diols having appropriate configurations result in the formation of cyclic esters, as shown in Equation 3. Although still hydrolytically labile, such cyclic esters possess sufficiently enhanced kinetic stability to shift the reaction equilibrium significantly to the right in aqueous solutions. Since this reaction produces a proton, reactions of boric acid with appropriate polyols can result in strongly acidic complexes. This reaction is commonly used in procedures for detection of boric acid and other boron compounds. In this method, a sugar, such as mannitol or sorbitol, is added to boric acid, and the resulting strong acid is titrated with a base, where a distinct endpoint characteristic of strong acids is observed.

$$B(OH)_3 + 3\,ROH \rightleftharpoons B(OR)_3 + 3\,H_2O \qquad \text{Eq. 2}$$



Eq. 3

R = H, alkyl, aryl

6

Boric acid reacts with two equivalents of diol to form spiroester complexes as shown in Equation 4. In cases where the diol is part of a polymer chain, this

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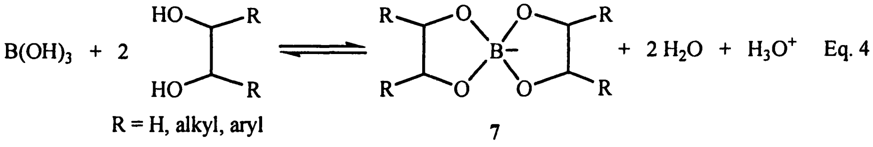
reaction leads to cross-linking. The addition of borates to aqueous solutions of appropriate hydroxylated compounds, such as certain sugars and starches, can greatly reduce pH.



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In the case of hydroxylated polymers, such as polyvinyl alcohols, guar gums, and related compounds, the addition of borates can have a dramatic effect on rheology and can form gels under the right conditions due to cross-linking between polymer chains. Synthetic borate esters produced by the reaction of boric acid with alcohols find use as components of many types of industrial fluids, including lubricants, hydraulic fluids, metal working fluids, and others. Borate cross-linking of polymers is utilized commercially in the manufacture of starch adhesives for corrugated boxes, in the formation of polyvinyl alcohol gel films to protect metal surfaces, and for other applications. The use of borate esters as intermediates in the manufacture of specialty chemicals and pharmaceuticals is becoming increasingly important.

Biological molecules frequently contain diol functionalities suitable for reversible ester formation with boric acid, and such interactions appear to be fundamental to the biological roles of boron. Interactions of boric acid with carbohydrates and other polyhydroxy compounds, possibly including proteins and glycoproteins, provide a basis for the biological actions of boron. It has been shown that one essential role of boron in plants involves cross-linking of the complex carbohydrate rhamnogalacturonan II (RG II) as part of a complex control mechanism vital to the maintenance of proper plant cell wall function (*9*).

The presence of adequate supplies of boron has been shown to be essential for the proper development of frogs and fish, and considerable evidence indicates that boron plays a vital role in other animals and humans (*10, 11*). Although precise biochemical mechanisms have yet to be revealed for animals, it appears likely that boron interacts with membrane components and enzymes in animal cells in significant and necessary ways through reversible ester formation.

Organoboron Derivatives

Although borate esters combine boron with organic moieties through B-O-C bond linkages, the term organoboron is more often applied to compounds containing boron-carbon bonds. These derivatives fall into a distinctly different class from the boron oxide derivatives discussed above. Whereas borate salts and esters readily convert to boric acid under physiological conditions, the B-C bonds in organoboron compounds are more resistant to hydrolytic cleavage under conditions typically found in biological systems. For this reason, organoboron derivatives may present different biocidal activities, as well as toxicological profiles, compared to boron oxides and esters. These differences in biological activity are largely dependent on the nature of the organic moiety attached to boron. Organoboron compounds are mostly specialty chemicals. Because they are more costly to manufacture, commercial organoboron derivatives generally have significantly higher prices than boron oxides or common boron esters.

Commercially-available organoboron compounds include trialkyl- and triaryl-boranes, BR_3 (R = alkyl, aryl), boronic acids, $RB(OH)_2$ (R = alkyl, aryl), and borinic acids, R_2BOH (R = alkyl, aryl). Other compounds containing boron combined with organic groups include oxazaborolidines, in which boron is complexed through B-O and B-N bonds. Many of these derivatives have been explored as biocides. Boronic acids, such as phenylboronic acid, form stronger complexes with diols than boric acid. For this reason, boronic acids tend to be stronger inhibitors of oxidoreductase enzymes due to their interactions with associated coenzymes, as discussed below. Phenylboronic acid and related compounds are effective wood preservatives but currently find little commercial use in this application due to their relatively high cost compared to the widely used and effective boron oxide wood preservatives (*12*). Trivalent boron compounds display Lewis acidity resulting in the ability to form adducts with electron donor species. These adducts can possess biocidal activity. For example, the triphenylboron-pyridine adduct has been used as an antifouling additive for coating applied to the underwater surfaces of ocean-going vessels. Oxazaborolidines have been shown to display antibacterial properties against *Streptococcus mutans* biofilm formation (*13*).

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Antimicrobial Properties of Boron Derivatives

Mode of Action

Like many antiseptics, boric acid is considered to have a non-specific mode of action because it attacks multiple targets in microbial cells. Boric acid is a small non-polar molecule capable of crossing biological membranes, which are permeable barriers separating the cell from its environment. The undissociated form of boric acid can interact with or pass through the membrane of the microbial cell and interfere with many enzymatic processes in the cell (*14*). It is believed that only the undissociated form of boric acid has the ability to cross cell membranes and exhibit any antimicrobial activity. In fact, the undissociate form of boric acid was more efficacious than its salts against Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* (*15*).

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Boron compounds can also react with hydroxyl-rich compounds such as phospholipids, lipopolysaccharides, and glycoproteins in microbial membranes. Such interactions could result in changes of membrane functional activity, including that of membrane-bound enzymes (*16*). The mode of action of boric acid can be linked to two possible major processes: inhibition of membrane proteins and inhibition of enzymes and co-enzymes inside cells. The inhibition of membrane proteins, including enzymes responsible for active transport, and interference with membrane structures may block nutrient transport and uptake and may slow down the metabolic processes of microorganisms. The inhibition of enzymes and co-enzymes inside cells could cause the dysfunction of metabolic pathways. Both processes could be the origins of the biostatic effect of boric acid on bacteria. Extended inhibition by either mechanism for more than 24 hours resulted in cell death (*15*).

It has been shown that boric acid inhibits at least two groups of enzymes: hydrolases (EC class 3) and oxidoreductases (EC class 1). Hydrolases that are known to be inhibited by boric acid include proteases, amylases (α-amylase EC 3.2.1.1; β-amylase EC 3.2.1.2), lipases (EC 3.1.1.3), cellulases, ureases (urea amidohydrolase EC 3.5.1.5), and β-lactamases (EC 3.5.2.6) (*17*, *18*). Affected oxidoreductases include alcohol dehydrogenases (EC 1.1.1.1), lactate dehydrogenases (EC 1.1.1.27) (*16*), peroxidases (EC 1. 11.), and aldehyde dehydrogenases (EC 1.2. 1.5).

The mechanisms by which boric acid inhibits hydrolases are complicated and have been intensively studied for the past two decades. It was reported that boric acid's stabilizing effect on proteases was due to reversible inhibition by boric acid and the polyborates (*18*). In fact, boric acid binds to the active catalytic site of subtilisin, a protease isolated from *Bacillus subtilus*. This binding involves interactions with serine, histidine, and aspartate at positions 221, 64, and 32, respectively, on the peptide chain (*18*). The ability of boric acid to temporarily block the active site of enzymes has been utilized in commercial liquid detergent formulations. For both consistent performance and acceptable shelf life, up to 3% of boric acid is often incorporated into liquid detergents. According to published data, in a liquid composition, proteases, amylases, lipases, and cellulases can be inhibited by any boric, boronic, or borinic acid derivative (*19*).

The urease phenotype is widely distributed across the bacterial kingdom, including pathogenic and soil microorganisms. Urease synthesis can be nitrogen-regulated, urea-inducible, or constitutive and requires the incorporation of both carbon dioxide and nickel ions into protein (20). It has been confirmed that boric acid acts as a competitive inhibitor of the ureases of *Proteus mirabilis* and *Klebsiella aerogenes* (*21*). The inhibition reached a maximum between pH = 6.2 and pH = 9.3, suggesting that only the neutral trigonal and not the tetrahedral borate anion acted as the inhibitor of urease. The authors reported the structure of the urease-boric acid complex that described the unique binding mode for boric acid to the active site and emphasized the role of the nickel ions in urea hydrolysis. The authors concluded that boric acid can be considered a substrate analogue, revealing the molecular details of the step of substrate binding (*21*).

The ability of bacterial strains to produce β-lactamase always contributes to antibiotic resistance. Kiener and Waley described reversible inhibition of the *Bacillus cereus* β-lactamase (class A enzyme) by boric acid and 3-aminophenylboronic acid (3-PBA) (*22*). Studies conducted by Waley demonstrated a similar inhibition mechanism when class C β-lactamases (also known as cephalosporinases) were involved.

An understanding of some details of inhibition of oxidoreductases by boric acid has developed during the past five years (*23-27*). The inhibition is based on a high affinity of boric acid for *cis*-diols and its ability to form ester complexes as outlined in Equation 3. It was shown that boric acid formed borate-ester complex with *cis*-diol-containing sugars, such as ribose and apiose, which play very important roles in cell structure and metabolism. Moreover, experiments showed that all NAD^+-, $NADP^+$-, and FAD-requiring oxidoreductases are competitively inhibited by borate added *in vitro* at ~0.4 mM. This inhibition is apparently due to the formation of borate esters with the *cis*-diol-containing ribose moieties of these coenzymes, as illustrated for NAD^+ in Figure 3 (*24-27*). Borate has a greater affinity for NAD^+ than NADH by a factor of 15, providing a selective control mechanism (*24-27*).

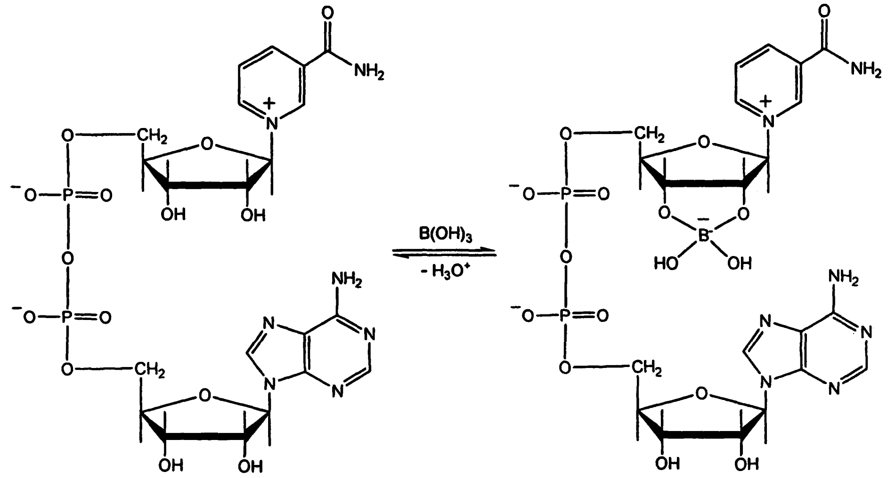

Figure 3. Proposed interaction of boric acid with NAD^+ resulting in inhibition of oxidoredastases.

The ability to react with ribose also provides an explanation for why boric acid binds to nucleotides. Boric acid acts as a reversible, pH-dependent inhibitor

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of Ca^{2+} uptake in cardiac microsomes (*26, 27*). Boric acid has a reversible effect on Ca^{2+} release, including Ca^{2+} uptake and regulation. It prevents the conversion of NAD to cADP. Boric acid also has the ability to affect cyclic compound formation (*26, 27*). Boric acid affects phosphate metabolism by inhibiting enzymes during oxidative phosphorylation and photophosphorylation.

In Vitro Susceptibility Studies of Boron Derivatives

Boric acid has proven to be a very effective and specific enzyme inhibitor and an influential factor in metabolism. It blocks the active sites of enzymes; however, its action is reversible. The antimicrobial effect of boric acid on microorganisms depends on the following factors: the critical role of inhibited enzymes (specific targets), period for which the repressed organism can survive without non-functional enzymes (exposure time), and how many enzymes and other primary functions of the organism are inhibited (concentration). Evaluations of these critical factors can be found in our original research.

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Antibacterial Efficacy of Boron Derivatives

The concentration of boric acid and its contact time with microorganisms play a critical role in its performance. New and previously published data has confirmed that boric acid is active against bacteria (Table I). Early studies by Novak and Taylor indicated that boric acid exhibits both bactericidal and bacteriostatic properties (*2*). Boric acid concentrations ranging from 0.5 to 2.0% provided bacteriostatic effects on typical Gram-positive bacteria often found in the eyes (Table I). Higher concentrations of 3.0 and 4.0% demonstrated bactericidal action after 3, 6, and 24 hours of exposure to boric acid.

A very extensive study describing the antimicrobial effect of boric acid on 161 standard and clinical strains associated with various infectious diseases was published in 1991 (*28*). The authors reported that the growth of all evaluated bacteria, including 10 Gram-positive and Gram-negative species, and fungi was completely inhibited by 1% (w/v) of boric acid added to the media. Bacterial isolates belonging to the same species showed similar minimum inhibitory concentrations (MICs) ranging between 0.125 and 1.00%. The authors compared MIC results for 60 *Staphylococcus aureus* strains with similar studies conducted 16 years earlier at the same hospital and found that all *S. aureus* strains, including methicillin-resistant *S. aureus* (MRSA), had not developed resistance to boric acid. Moreover, there were no differences in boric acid MICs between methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA). The effect of boric acid on MRSA suggested that use at appropriate dosages may be beneficial in treating bacterial skin infections (*28*).

Table I. Antibacterial Activity of Boric Acid

Organism	Concentration (%)	Contact Time (hours)	Test Method	Ref.
Micrococcus pyogenes var. aureus	4.0-5.0	48	1	*29*
Micrococcus pyogenes	2.0-4.0	3-24	2	*2*
Streptococcus sp. (β-hemolytic)	4.0 2.0-3.0	3-24 24	2	*2*
Diplococcus pneumonie	4.0 1.0-3.0	3-24 24	2	*2*
Staphylococcus aureus ATCC 6538	0.12-0.25	24	3	*15*
Streptococcus sp.	1.0-2.0	24	3	*7*
Pseudomonas aeruginosa	1.0-2.0	24	3	*7*
Pseudomonas aeruginosa ATCC 15442	0.25-0.50	24	3	*15*
Escherichia coli ATCC 11229	0.25-0.50	24	3	*15*
Acinetobacter calcoaceticus	1.0-2.0	24	3	*7*

Test methods: (1) zone of inhibition, (2) infusion broth, plate counts, (3) MIC-nutrient broth dilution.

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In 1969, Porter and Brodie described how boric acid at a concentration of 1.8% could be used to preserve urine samples while in transit for bacteriological examination (*7*). The authors showed that when boric acid was added to urine, the total number of bacteria present did not change significantly for up to 48 hours at room temperature. The effectiveness of boric acid for preserving urine before its bacteriological examination was later confirmed by Lum and Meers (*30*). Boric acid, at a concentration of 20 g/L, exhibited bacteriostatic effects, eliminating the false positive results obtained with unpreserved specimens; the effect lasted for up to 48 hours (*30*). In a follow-up study, 17 bacterial and fungal species commonly found in urine were exposed to different concentrations of boric acid in nutrient broth at room temperature and examined at four- and six-hour intervals for up to 24 hours to detect bacteriostatic and bactericidal effects (Table I) (*7*). Boric acid at 10 g/L was weakly bactericidal for some strains of *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa*. At concentrations between 10.0 and 20.0 g/L, boric acid exhibited bacteriostatic and fungistatic effects for nearly all the common urinary pathogens, including Streptococcus group B strains; however, the majority of *A. calcoaceticus* and *P. aeruginosa* strains required concentrations of 20.0 g/L and higher.

Borokhov studied the antimicrobial effects of boric acid on the growth of Gram-positive *S. aureus* and Gram-negative *E. coli* and *P. aeruginosa* bacteria. The author reported that the antimicrobial effective concentrations of boric acid were specific to each evaluated organism and ranged from 0.40% for *S. aureus* to 1.25% for *P. aeruginosa*; however, the action mechanism was similar for all three species (Figure 4, A-C). Within the first 24 hours of exposure, boric acid provided bacteriostatic effects against *P. aeruginosa* (Figure 4C) and moderate bactericidal effects (2-log reduction in microbial populations) against *S. aureus* and *E. coli* (Figures 4A and 4B). Continuous exposure of microorganisms to boric acid for 48 hours resulted in reductions of microbial populations for *S. aureus* (4-log) and *E. coli* (3-log). Extended exposure of up to 7 days provided strong biocidal effects with more than 99.999% kill for all three evaluated microorganisms. Gram-positive bacteria were more susceptible to boric acid than Gram-negative bacteria. For example, boric acid at a concentration of 0.40% provided a bactericidal effect against Gram-positive *S. aureus* within 24 hours with a 6-log reduction after continuous exposure for 7 days (Figure 4A). In contrast, higher levels of boric acid, such as 0.80% and 1.25%, were required to control Gram-negative *E. coli and P. aeruginosa* and to achieve 6-log reduction in microbial populations (Figures 4B and C). Lower concentrations provided bacteriostatic effects, which gradually became bactericidal after 7 days of exposure time (*15*).

In contrast to boric acid's performance under extended contact times, boric acid has not provided bactericidal effects after a very short contact time, such as 30 seconds, on neither Gram-negative nor Gram-positive bacteria. Boric acid was evaluated among thirteen antiseptic solutions intended for disinfection of living tissue against *P. aeruginosa* and *S. aureus*. Under sanitizer test conditions (*in vitro* study, 10^7 cfu/mL, 30 sec and 120 sec contact time), 3.5 % boric was not effective against either organism (*31*).

In addition to boric acid, several other boron derivatives exhibit antimicrobial properties. The efficacy of borax decahydrate was evaluated against four bacterial species, *Bacillus subtilis*, *Cellulomonas sp.*, *Erwinia carotovora*, and *Serratia marcescens*, which are able to colonize wood and degrade wood components (*32*). The lethal dose of 50 (LD_{50}), which was defined as the decrease of living cells of $\geq$ 50%, and the lethal dose of 100

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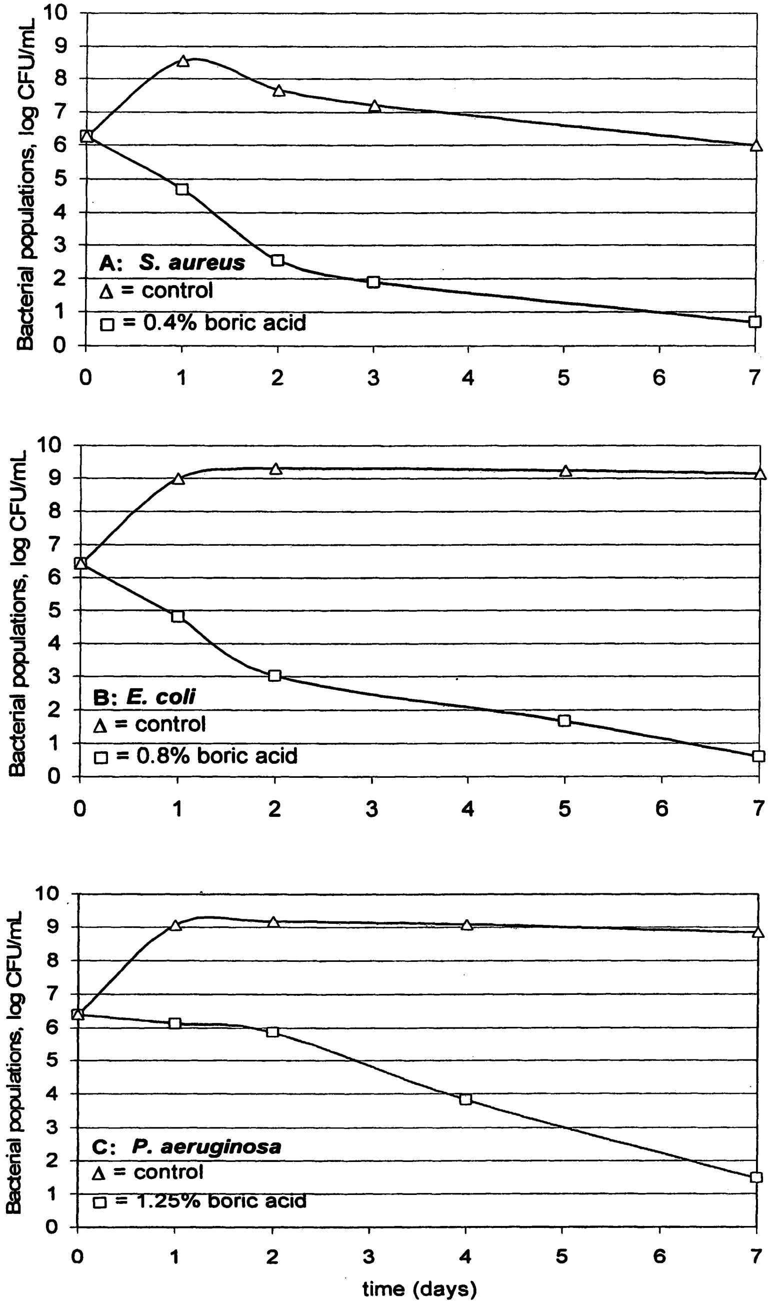

*Figure 4. Antimicrobial effects of boric acid on the growth of Gram-positive S. aureus (A), Gram-negative Escherichia coli (**B**), and P. aeruginosa bacteria (**C**). Temp. = 37°C.*

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(LD_{100}), which was defined as a 100% decrease within 24 hours of incubation, were used as criterions for evaluation. Experimental results in a medium free of nutrients showed that a concentration of borax decahydrate from 0.1 to 1.0% w/w is required to decrease 50% of microbial populations for all four species. The complete bactericidal effect (LD_{100}) was achieved for *Cellulomonas sp.* and *S. marcescens* at 1.0% w/w and *E. carotovora* at 5.0% (Table II). *B. subtilus* was the most resistant among the four bacteria; it required concentrations of more than 5% and tolerated sub-lethal concentrations by sporulation.

Table II. Antibacterial Activity of Borax ($Na_2B_4O_7•10H_2O$)

Organism	Concentration (%)	Contact Time (hours)	Test Method	Ref.
Bacillus subtilis W23	> 5.0	24	LD_{100} Aqueous mineral solution	*32*
Cellulomonas sp. DSM	1.0	24	LD_{100} Aqueous mineral solution	*32*
Erwinia carotovora CCM	5.0	24	LD_{100} Aqueous mineral solution	*32*
Serratia marcescens DSM 47	1.0	24	LD_{100} Aqueous mineral solution	*32*
Pseudomonas aeruginosa ATCC 15442	0.5–1.0	24	MIC - nutrient broth dilution	*15*
Escherichia coli ATCC 11229	0.5–1.0	24	MIC - nutrient broth dilution	*15*

Studies of the effect of different media on the sensitivity of bacteria showed that bactericidal concentrations need to be higher in nutrient liquids than in water (*32*).

Antimicrobial properties of boron derivatives against two species of cyanobacteria (blue-green algae), *Gloeocapsa alpicola* 1598 and *Gloeocapsa sp.* 795, were evaluated in aqueous solution for 21 days under a light-dark cycle of 12-12 hours. Samples containing boron derivatives at 0.06 and 0.1%, expressed as boric acid, were clear and colorless, indicating an absence of photosynthetic pigments, while the control sample had a deep green color. The plate count method showed a reduction in bacterial populations and the development of slow-growing colonies requiring extended cultivation time. The results of this study showed that the combination of boric acid and borate tetrahydrate at concentration of 0.06% and 0.1%, expressed as boric acid, completely inhibited the multiplication and photosynthesis of *G. sp.* and *G. alpicola* (*15*).

Boric acid esters derived from various alcohols were prepared and screened for antimicrobial activity in spent coolants of water-based cutting fluids by Watanabe's group (*33*). Boric acid esters of hydroxyl amines (amino alcohols), such as 2-aminobutanol, 2-(2-aminoethyoxy)ethanol, 3-amino-2,2-dimethyl-1-propanol, and 5-aminopentanol, at concentrations of 3% w/w, showed good antimicrobial activity. Boric acid esters of di- and tri-ethylene glycols at concentrations of 3% w/w showed biostatic properties. The test system consisted of 3.0% of boric acid esters, standard broth medium, iron chips, and sterile water. Inoculum was 1.0 g samples of commercially available spent coolant contaminated with a variety of bacterial species. Samples were incubated at 35°C for 4 weeks. Every three days, the samples were re-inoculated using 1.0 g of cutting fluid (spent coolant). After 30 days of incubation, the samples had no microbial growth, leading to the conclusion that boric acid esters of hydroxylamines exhibited excellent bactericidal properties (*33*).

Watanabe continued to evaluate the antimicrobial properties of reaction products of a variety of aminoalcohols with boric anhydride in spent coolant of water-based cutting fluids. The products of the reaction of lower alcohols with boric anhydride showed antimicrobial activity against natural contaminations, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas olevorans*, *Staphylococcus aureus*, *Desulfovibrio desulfuricans*, and *Fusarium sp.*, in spent coolant (*34*).

Borate esters derived from various alcohols are highly effective for controlling microorganisms in all hydrocarbon fuels, including diesel and kerosene, and have been used as gasoline additives since the mid-60s (*35*, *36*). *Biobor* J.F., a mixture of two borate esters, 2,2'-(1-methyltrimethylenedioxy)-bis(4-methyl-1,3,2-dioxaborinane) and 2,2'-oxybis(4,4,6-trimethyl-1,3,2-dioxane), provided effective antimicrobial control against a mixture of bacteria and fungi at 270 ppm in jet fuel (*35*, *36*). Complete kill against a moderate level of mixed bacteria and fungi ($5 \cdot 10^4$cfu/mL) was achieved within 72 hours; however, longer treatment time was required against higher concentrations ($1 \cdot 10^6$–$1 \cdot 10^9$ cfu/mL) of microorganisms (*36*). In order to maintain microbiological control, the continuous use of 135 ppm of *Biobor* has been recommended, especially in case of possible re-inoculation of fuel tanks (*37*). DeGray and Fitzgibbons investigated the rate of kill and the action mechanism of organoborates in a two-phase system consisting of hydrocarbon fuel and water (*16*). Borate esters kill hydrocarbon-ingesting microorganisms with a lower concentration of boron than does boric acid. In an aqueous solution, there are no benefits of organoborates over boric acid because organoborates are hydrolyzed to boric acid and organic alcohol (*16*).

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Efficacy of Boron Derivatives Against, Fungi, Algae, and Protozoa

The *in vitro* antifungal activity of boric acid was evaluated by the Otero group using three different test methods (*3*). Thirty-seven *Saccharomyces visiae*, 39 *Candida* non-*C. albicans* isolates, and one *Trichosporon sp.* isolate were evaluated using different susceptibility testing methods: agar dilution methods with high and low inoculum concentration and a standard broth microdilution method (National Committee for Clinical Laboratory Standards document NCCLS M-27A) (*3*). The boric acid MICs obtained by the agar dilution and broth microdilution methods are shown in Table III. The susceptibility range for all tested yeasts exposed to boric acid fell within 0.05% and 1.60%. The MICs_{90} showed similar results regardless of the method used and ranged between 0.40 and 1.60% (Table III). The efficacy of boric acid also depended on the inoculum concentration.

The mechanism of antifungal activity of boric acid was evaluated against eight *Candida* vaginal isolates (six *C. albicans*, two *C. glabrata*) by the Rodrigues group (*40*). Fungistatic activity was assessed as MIC using the microdilution method (National Committee for Clinical Laboratory Standards document NCCLS M-27A); fungicidal activity was determined as minimal lethal concentration (MLC) using viability plate counts. Boric acid demonstrated fungistatic activity at 0.39 mg/mL for all tested strains except for *C. glabrata*, which required 1.56 mg/mL. Higher concentrations ranging from 4 to 16 times the MIC were required to achieve fungicidal effects, with the exception of *C. glabrata*, which required a 32-fold increase (*40*).

The *in vitro* activity of antifungal and antiseptic agents, including boric acid, was evaluated against dermatophytes isolated from patients with *tinea pedis* (39). The MIC or the minimal dilution concentration (MDC) was determined by an agar dilution method using a modified yeast nitrogen agar base, and the minimum fungicidal concentration (MFC) or minimum fungicidal dilution (MFD) was determined with subcultures on Sabouraud dextrose agar (Table III). The effective concentration of boric acid ranged from 512 mg/L to 0.1 mg/L. All agents, including boric acid, were active against the dermatophytes at lower concentrations than those used in products and topical pharmaceutical formulations. It was concluded that the use of these antiseptic agents represents an excellent alternative for the topical treatment of *tinea pedis*.

The algaecidal and fungicidal activity of boron derivatives was tested using the pure culture EPA method (*38, 41*). Two algal *Chlorella pyrenoidosa* and *Chlorococcum mustard* test organisms and the fungi *Aspergillus flavus* were evaluated in an aqueous solution at 22 to 24°C for three weeks. The authors reported that the addition of 25 mg/L of sodium tetraborate pentahydrate completely inhibited any *Ch. mustard* growth, while at least 200 mg/L and 48 hours of contact time were required to control the two algae species *Ch. pyrenoidosa* and *Ch. mustard* and the fungi *A. flavus* (*38*, *41*).

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Table III. Antimicrobial Efficacy of Boron Derivatives for Fungi and Algae

Organism	Derivative	Concentration	Contact Time	Test Method	Ref.
Chlorococcum mustard	$Na_2B_4O_7 \cdot 5H_2O$	25 mg/L	3 weeks	1	*38*
Chlorella pyrenoidosa	$Na_2B_4O_7 \cdot 5H_2O$	200 mg/L	3 weeks	1	*38*
Aspergillus flavus	$Na_2B_4O_7 \cdot 5H_2O$	200 mg/L	3 weeks	1	*38*
Saccharomyces cerevisiae (4)	$B(OH)_3$	0.4-1.6%	48 hours	2	*3*
Candida glabrata (*39*)	$B(OH)_3$	0.4-1.6%	48 hours	2	*3*
Candida parapsilosis (*35*)	$B(OH)_3$	0.4-0.8%	48 hours	2	*3*
Trichophyton mentagrophytes (*27*)	$B(OH)_3$	64->512 mg/L 512->512 mg/L	5-7 days	3	*39*
Trichophyton rubrum (*39*)	$B(OH)_3$	64->512 mg/L 512->512 mg/L	5-7 days	3	*39*
Epidermophyton floccosum (*29*)	$B(OH)_3$	128->512 mg/L 512->512 mg/L	5-7 days	3	*39*

Test methods: (1) EPA TSD 6.101, (2) MIC_{90} by agar dilution and broth microdilution, (3) MIC agar dilution method NCCLS M-27A MFC subculture on agar plates (SDA).

The antimicrobial effectiveness of novel N_2B heterocycles that are formed from the reaction of ethylene diamine derivatives with 2-(4,4,5,5,-tetramethyl-1,3,2,-dioxaborolan-2-yl)benzaldehyde (2-HC(O)C_6H_4Bpin; pin=1,2-$O_2C_2Me_4$) was recently reported by Irving's group (*42*). All of the new compounds showed considerable antifungal activity against *Aspergillus niger* and *Aspergillus flavus* and moderate antibacterial activity against *Bacillus cereus*.

Boron derivatives have been used for the preservation of wood against wood decay fungi for several decades (*43*). Both white-rot and brown-rot fungi are susceptible to boric acid and its salts. The effective preservative levels range from 0.1 to 1.0% w/w and depend on the specific kind of timber, fungi, and test methods.

Borates are effective against several protozoa species isolated from Formosan subterranean termite guts. It was shown that borate solution reduced termite gut protozoan populations. In forced-feeding laboratory studies, Douglas fir heartwood treated with boric acid resulted in 100% mortality within three weeks (*44*).

Antimicrobial Properties of Formulations Containing Boron Derivatives

Several papers reported that the presence of boric acid in antimicrobial formulations can enhance performance and significantly reduce the amount of another active ingredient. Based on these synergistic properties, boric acid is widely used in different applications.

In a study conducted by Amicosante's group, boric and boronic acids were investigated as inhibitors of β-lactamases produced by clinical isolates: two *Citrobacter diversus* strains and a *Pseudomonas aeruginosa* strain (*17*). The β-lactamases produced by the two *C. diversus* strains growing on cephazolin as the substrate were inhibited by both borates and boronates. The enzyme from *P. aeruginosa* growing on benzylpenicillin as the substrate was inhibited only by boronates. These inhibitors were also used in combination with selected β-lactamases to identify synergistic effects. Results indicated that the minimum inhibitory concentration (MIC) values were lowered in the presence of boric and boronic acids for the two *C. diversus* strains. In contrast, the MIC values for the *P. aeruginosa* strain were not significantly altered, thus indicating the presence of a permeability barrier for 3-aminophenylboronic acid.

Several ophthalmology-related studies confirmed the beneficial role of boric acid as an effective synergist. A minimal salts medium adjusted to physiological pH and osmolality was buffered with either 0.3% phosphate or 1.2% borate and evaluated for antimicrobial activity in a challenge test (USP XXI) (*45*). Borate-buffered media, with or without a carbon source, exhibited significant antimicrobial activity against 15 *Pseudomonas* strains, 12 enteric bacteria strains, and three staphylococci strains. The borate-buffered system appeared to be beneficial for use as a generic vehicle for ophthalmic agents.

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Koshiro and Oie evaluated eye drop formulations containing boric acid and parabens (*46*). While both methylparaben and propylparaben exhibit only moderate bactericidal activity, they were used as active ingredients at low levels. The antimicrobial activity of the preservative formulation, comprising a conventional solvent, 0.026% of methylparaben (methyl p-hydroxybenzoate), 0.014% of propylparaben (propyl p-hydroxybenzoate), and 2.0% of boric acid, was tested against nine strains of *Pseudomonas aeruginosa* and one strain each of *Pseudomonas cepacia*, *Pseudomonas maltophilia*, *Escherichia coli*, and *Staphylococcus aureus*. Due to the additive effects of boric acid, the preservative formulation showed strong antimicrobial activity against eight out of nine strains of *P. aeruginosa*. However, the antimicrobial activity of the preservative formulation was not effective against one of the strains of *P. aeruginosa*, *P. cepacia*, *E. coli*, and *S. aureus*.

The benefits of a borate buffer in a hydrophilic contact lens solution were investigated by Arora's group (*47*). The antimicrobial properties of a multipurpose solution, which contained 0.0001% Polyhexanide, a surfactant, a sequestering agent, and one of three different buffer systems (borates, citrates, and phosphates), were evaluated against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* in a zone of inhibition test. Results showed that the borate formulated product at pH = 7.45 provided the best efficacy against all three microorganisms.

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Toxicology

Boron occurs naturally throughout the environment and is essential for plant life. As a result, boron is a natural part of foodstuffs and a component of a healthy human diet. Under physiological conditions, inorganic borates, including boric acid, borax, and other sodium borates, and NH_4^+, K^+, and Zn^{2+} salts all dissociate to boric acid. The US Environmental Protection Agency regulates the use of inorganic borates as pesticides under the authority of two federal statutes: the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). More recently, the Food Quality Protection Act of 1996 (FQPA) amended FIFRA and FFDCA by setting tougher safety standards for new and old pesticides (*48*). Common inorganic borates display low acute toxicities in mammalian species (*49*). In fact, for most species, borates' acute oral LD_{50} values vary from 2000 to 5000 mg/kg (*50*). Reported boric acid acute oral toxicity was from 2660 to 4000 mg/kg in rats and 3450 and 2000 mg/kg in mice and dogs, respectively (*50*). The acute dermal LD_{50} in rabbits was greater than 2000 mg/kg for majority of inorganic borates (*50*). Toxicological studies showed that with regard to human exposure, these compounds are not skin irritants or skin sensitizers and have no mutagenic or carcinogenic effects (*49, 50*). Other studies employing high chronic exposure levels have shown developmental toxicity and

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male reproductive system effects in several mammalian species, with similar toxicological effects across species. These data are important to consider with regard to the proper uses of borate products. However, their toxic thresholds are considerably higher than humans are likely to be exposed under normal handling and use (*49*, *51*). Thus, borates are regarded as safe ingredients for use in consumer products, including ophthalmologic solutions, topical ointments, lotions, and soaps. They are not generally allowed for use as intentional food additives. However, the FDA has approved boric acid and borax decahydrate for use in the production of paper under the indirect food additive regulations of 21 CFR.

Biological Importance of Boron

Boron has been recognized as an essential micronutrient for plants since the 1920s (*52*). All plants contain measurable levels of boron, and deficiency of boron may be the most prevalent agricultural micronutrient deficiency problem in the world (*53*). Thus, it is standard agricultural practice to apply boron to crops in areas where boron soil concentrations are suboptimal for particular crops, typically by using borates mixed with other required fertilizers. Although much is still unknown, considerable progress has been made recently in understanding the biological role of boron in plants. In particular, boron is now known to play an essential role in plant cell wall function through ester cross-linking with complex carbohydrates (*9*). Boron concentrations in plants and plant-derived foodstuffs are typically less than 100 ppm, but can range up to a few thousand ppm (*54*, *55*).

There is now substantial evidence that boron is also biologically important to animals and humans (*10*, *11*). Consumption of plants by herbivorous animals introduces boron into the food chain, and animals and humans inevitably contain low levels of boron. For example, human blood typically contains 0.01-0.17 $\mu g \cdot mL^{-1}$ and urine contains 0.15-2.98 $\mu g \cdot mL^{-1}$ (*55*). Body tissues have boron concentrations comparable to those in blood, and bone may contain somewhat higher levels. Ingested boron is primarily excreted in the urine within approximately 24 hours. It is estimated that the average adult in the USA consumes approximately 1 mg of boron per day, and boron consumption by healthy populations in other parts of the world can be considerably higher, depending on regional diets (*56*).

Conclusions

Boron derivatives exhibit broad-spectrum antimicrobial activity. *In vitro* biocidal activity have been shown for Gram-positive and Gram-negative bacteria, yeast, and fungi (Tables I, II, III). Analysis of existing literature and

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new data indicates that Gram-positive bacteria are less tolerant and more susceptible to boric acid than Gram-negative bacteria. Within 24 hours of contact, the effect of boric acid on Gram-negative bacteria is bacteriostatic rather than bactericidal (Table I). This occurs because the ability of borates to cross the cell wall and membrane and act inside cells is somehow limited due to the difference in the chemical composition of the cell walls. While boric acid's antimicrobial effective concentration is very specific, the extended exposure can achieve excellent bactericidal effects against both Gram-positive and Gram-negative bacteria (Table I) *(15)*.

The contact time between microorganisms and the antimicrobial agent is a critical factor in the agent's performance. Analysis of existing literature and new data suggests that boric acid is a slow-acting agent. Therefore, in order for it to achieve a biocidal effect, microorganisms should be exposed to boron derivatives for a long period of time. In fact, a minimum of 3 hours *in vitro* contact time is required for reducing microbial populations of typical eye infections, and an extension to 24 hours provided a bactericidal effect with complete kill of bacteria (Table I). In other cases, 24 hours of contact time at a concentration of 1.0% and 2.0% of boric acid provided only a bacteriostatic effect against urinary pathogens (*7*). Although yeasts are susceptible to boric acid, the fungicidal effect could be achieved within several days (Table III). Filamentous fungi also need extended exposure to be completely inhibited by boron derivatives. Some organisms, including spore-forming bacteria and fungi, can tolerate sub-lethal concentrations by sporulation (*31*).

Compatibility of boric acid with the medium also affects the rate of transport of borates across the cell membrane. Ingredients present in the medium can enhance or inhibit the performance of boron derivatives (*16*). In fact, the lipophilicity of borate in the presence of a hydrocarbon phase was critical in achieving the biocidal efficacy of *Biobor* JF in fuels (*16*).

Boric acid can be a very effective antimicrobial agent, especially when it is not expected to provide disinfectant properties and applications. Based on its good environmental profile and broad-spectrum activity against bacteria, yeast, and fungi, it is suitable for many medical and industrial applications. Due to the inhibition action mechanism of boron compounds, extended contact time is required to achieve bactericidal effects. Therefore, boron compounds perform better in preservative-type efficacy tests.

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Chapter 21

Comparison of Mycobacterial Susceptibilities to Six Chemical Disinfectants

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Objective: To compare the mycobacterial susceptibilities among 10 mycobacteria to six disinfectants. [Methods] 10 mycobacterial species and 6 disinfectants were used in this study. We first set up a quantitative suspension mycobactericidal test in the light of Chinese technique standard for disinfection, and then we used the test to evaluate the susceptibilities of each mycobacterium to the tested disinfectants. [Results] The 10 mycobacteria are similarly susceptible to glutaraldehyde, peracetic acid, ethanol and cresol disinfectants, M. fortuitum and M. chelonei subsp. abscessus are more resistant to chlorine disinfectant than the other 8 tested mycobacteria. [Conclusion] M. fortuitum and M. chelonei subsp. abscessus were more resistant than or at least as resistant as the other tested mycobacteria to disinfectant.

1. Introduction

Determinations of the incidence of tuberculosis word-wide are notoriously difficult, but according to World Hearth Organization (WHO) estimates, one-third of the world's population has been infected, about 100 million individuals are infected annually (1), non-tuberculosis mycobacteria (NTM) infection has been increasing rapidly since 1980's (2-3). Disinfection is an effective way to control mycobacterial infection. Because of the high lipid content in their cell walls, mycobacteria are more resistant to disinfectants than other bacterial vegetatives, hence the disinfectants used to prevent and control mycobacterial infection should be selected carefully.

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2. Materials and methods

2.1 Strains and materials

2.1.1 Mycobacterial strains

In this study, 10 species of mycobacteria were used, namely M. tuberculosis H37Ra CMCC (B) 93020, M. bovis CMCC (B) 93006, M. nonchromogenicum CMCC (B) 93311, M. xenopi CMCC (B) 93316, M. phlei CMCC (B) 93318, M. smegmatis CMCC (B) 93203, M. diernhoferi CMCC (B) 93320, M. flavescens CMCC (B) 93322, M. fortuitum CMCC (B) 93323 and M. chelonae subsp. abscessus CMCC (B) 93326.All mycobacteria were provided by Chinese Medical Culture Collection of Bacteria [CMCC (B)].

Among the 10 tested mycobacteria, M. chelonae subsp. Abscessus, M. fortuitum, M. smegmatis, M. phlei, M. diernhoferi and M. flavescens are rapid growing mycobacteria which can grow to be seen on the media within 7 days, the other 4 are slowly growing mycobacteia.

2.1.2 Disinfectants

The six disinfectants used in this research were ERIC (an iodophor based disinfectant, manufactured by Chengdu Yongan Pharmacia CO), GA-50 (a glutaraldehyde based disinfectant, manufactured by Huaxi Center of Health Care Science and Technology), TC-101disinfection tablet (a chlorine based disinfectant, manufactured by Chinese PLA 7018 factory), Ethanol (manufactured by Neijiang Kanghong sanitary articles factory), Peracetic acid and Ethanol. Peracetic acid (PAA) was prepared from hydrogen peroxide and acetic acid in our own laboratory as described previously. Alcohol was prepared by diluting dehydrated ethanol with sterile distilled water.

Tested concentrations of each disinfectant were prepared by diluting with sterile distilled water.

2.1.3 Neutralizers

In this study, 0.03mol/L phosphate buffer solution (PBS) containing 1% tween 80, 1% lecithin and 1% glycine was used as neutralizer to glutaraldehyde disinfectant, 0.03mol/L PBS containing 0.5% tween 80 and 1% sodium thiosulfate to iodine, chlorine and peracetic acid disinfectants and 0.03mol/L PBS containing 0.5% tween 80 and 1% lecithin to cresol and ethanol disinfectants.

All the neutralizers, according to Chinese technique standard for disinfection, were testified effective to stop the lasting effect of their corresponding disinfectants and harmless to the tested mycobacteria.

2.2 Mycobactericidal Tests

Tested mycobacteria were incubated on slants of Mycobacteria Dehydrate Media at 37°C for 4 days and 20~30 days respectively for rapid growing mycobacteria and slow growing ones (M. xenopi incubated at 42°C for 20 days). Add 3~5 ml dilution (0.03mol/L phosphate buffer solution, pH 7.2) on to the slants and scrape the culture gently with a sterile incubation loop into the dilution. Then the mixture of dilution and culture were transferred to a small sterile flask containing glass pieces, shake vigorously for 5~10 min. Dilute the suspension to 107~108cfu/ml with sterile dilution.

Pipette 5 ml disinfectant with different concentration into a sterile tube, incubate 5 min within 20~22°C water bath, and then add 0.1 ml of the tested mycobacterial suspension (107~108cfu/ml) into each of the tubes, vortex for 5 sec. After being exposed for selected time, 0.5 ml of the mixture was transferred to 4.5 ml sterile neutralizer (testified to neutralize the lasting effect of the corresponding tested disinfectant). After 10 minutes' neutralization, 0.2 ml of the mixture was inoculated onto the surface of L-J media plate and then incubated for viable count. The recovery cultural conditions (time, temperature etc) for each of the tested mycobacteria are consistent with the conditions mentioned above.

Mycobacterial control plates were designated with exactly performance same as the tested plates with the exception of substituting of disinfectant with sterile water. Killing rate (KR) was calculated by the following formula.

$$KR = \frac{Nc - Nt}{Nc} \times 100\%$$

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In the formula, Nc means viable count of mycobacterial control (cfu/ml), and Nt means viable count of each bactericidal test (cfu/ml).

3. Results

Results in Table 1 showed the bactericidal results of ERIC against the 10 tested mycobacteria. Data in this table suggest that the 10 tested mycobacteria would be killed by more than 99% after exposing to 40mg/L available iodine for 1 min. With the exposing time being prolonged, the killing rates are increasing. No tested mycobacteria survive after exposing to 40mg/L available iodine for 20 minutes.

The killing rates of iodine disinfectant against M. chelonei subsp. abscessus are almost always lower than the other 9 myocbacteria when exposing to the same iodine concentration for the same time, which indicate that M. chelonei subsp. abscessus is comparatively less susceptible to iodine disinfectant than the other 9 tested mycobacteria.

Table 2 to Table 5 showed the bactericidal results of glutaraldehyde, cresol, peracetic acid and ethanol against tested mycobacteria respectively. For each of these 4 disinfectants, the killing rates of the same concentration and exposing time are very similar among the 10 tested mycobacteria, which indicate that these 10 mycobacteria are similarly susceptible to glutaraldehyde, cresol, peracetic acid and ethanol disinfectant.

The bactericidal results of chlorine disinfectant are listed in Table 6. TC-101 solution with 40 mg/L available chlorine can kill M. tuberculosis H37Ra, M. bovis, M. nonchromogenicum, M. xenopi, M. phlei, M. smegmatis, M. diernhoferi and M. flavescens by more than almost 100% after exposing for 20 min, while 80 mg/L available chlorine is needed to kill M. fortuitum and M. chelonae subsp. abscessus by the same percentage after exposing for the same 20 min, which indicates, in order to acquire the same killing rate when exposing for the same time, M. fortuitum and M. chelonae subsp. abscessus need double dosage of available chlorine as the other 8 tested mycobacteria.

4. Conclusion and Discussion

It is well accepted that mycobacteria are more resistant to disinfectants than most other bacterial vegetatives, owing to their high lipid contents in cell walls and complex cell wall structures. In order to guarantee the disinfection effect against mycobacteria, the disinfectants and disinfection procedures must be testified by a mycobactericidal evaluation test. Most countries and organizations have issued their own authoritative mycobactericidal evaluation tests, however, these tests differ in test strains, operation procedures and evaluation standards.

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Table 1. Bactericidal efficacy of ERIC against test mycobacteria

Test mycobacteria	Average killing rate (%) of available iodine concentration (mg/L) after different exposing time (min)											
	40 mg/L				20 mg/L				10 mg/L			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min
M. phlei	99.94	99.99	100	100	98.73	99.88	99.99	100	81.64	96.98	99.27	99.91
M. fortuitum	99.93	99.99	100	100	98.60	99.84	99.98	100	76.94	95.86	99.13	99.79
M. smegmatis	99.61	99.96	99.99	100	96.87	99.72	99.95	99.99	81.82	97.18	99.10	99.82
M. diernhoferi	99.97	99.99	100	100	98.53	99.80	99.98	99.99	73.59	96.07	99.29	99.86
M. chelonae subsp. abscessus	99.39	99.95	99.99	100	89.00	98.28	99.66	99.96	75.30	90.59	98.39	99.66
M. flavescens	99.58	99.93	99.99	100	96.41	99.50	99.93	99.99	77.44	95.30	98.99	99.78
M. nonchromogenicum	99.98	99.99	100	100	98.87	99.88	99.99	99.99	78.66	95.80	99.16	99.85
M. tuberculosis H37Ra	99.65	99.98	100	100	95.75	99.33	99.87	99.99	91.02	96.66	99.37	99.86
M. xenopi	99.66	99.97	100	100	96.63	99.42	99.92	99.99	81.72	96.35	99.15	99.85
M. bovis	99.61	99.98	99.99	100	95.20	99.28	99.90	99.99	81.66	95.78	98.65	99.76

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Table 2. Bactericidal efficacy of GA-50 against test mycobacteria

Test mycobacteria	Average killing rate (%) of glutaraldehyde concentration after different exposing time (min)											
	1%				0.5%				0.25%			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min
M. phlei	99.93	99.99	100	100	99.46	99.96	99.99	100	85.44	98.00	99.59	99.94
M. fortuitum	99.93	99.99	99.99	100	99.31	99.92	99.99	100	82.96	97.51	99.11	99.92
M. smegmatis	99.99	100	100	100	99.85	99.99	100	100	89.86	99.89	99.96	99.97
M. diernhoferi	99.95	99.99	100	100	98.82	99.92	99.99	100	83.29	95.25	99.35	99.92
M. chelonae subsp. abscessus	99.93	99.99	100	100	99.24	99.94	99.99	99.99	83.10	97.30	99.44	99.93
M. flavescens	99.98	99.99	100	100	99.71	99.95	99.99	100	88.82	99.26	99.88	99.98
M. nonchromogenicum	99.93	99.99	100	100	99.31	99.91	99.99	100	84.53	97.24	99.22	99.92
M. tuberculosis H37Ra	99.92	99.99	99.99	100	99.34	99.92	99.99	100	90.76	98.11	99.68	99.94
M. xenopi	99.92	99.99	99.99	100	99.34	99.92	99.99	100	90.76	98.11	99.68	99.94
M. bovis	99.96	99.99	100	100	99.38	99.93	99.99	100	83.26	96.71	99.43	99.93

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Table 3. Bactericidal efficacy of cresol against test mycobacteria

Test mycobacteria	Average killing rate (%) of cresol concentration (%)after different exposing time (min)											
	10%				7.5%				5%			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min
M. phlei	99.73	99.99	100	100	86.00	99.14	99.94	99.99	54.43	85.42	97.80	99.76
M. fortuitum	99.59	99.99	100	100	81.22	99.52	99.93	99.99	40.00	80.84	97.01	99.71
M. smegmatis	97.69	99.98	100	100	66.00	95.80	99.50	99.98	50.94	77.36	93.61	97.96
M. diernhoferi	99.77	99.98	100	100	86.81	99.01	99.94	99.99	64.69	84.02	98.25	99.79
M. chelonae subsp. abscessus	99.94	99.97	99.99	100	80.16	99.36	99.94	99.99	55.35	83.32	97.75	99.63
M. flavescens	98.69	99.93	99.99	100	69.87	95.77	99.41	99.94	54.68	76.54	93.03	97.71
M. nonchromogenicum	99.80	99.98	100	100	87.11	99.52	99.94	99.99	53.45	83.77	97.39	99.72
M. tuberculosis H37Ra	99.98	99.99	100	100	95.62	99.37	99.92	99.99	59.53	82.78	94.45	98.72
M. xenopi	99.98	100	100	100	98.38	99.81	99.98	99.99	67.08	89.83	97.20	99.61
M. bovis	99.99	100	100	100	98.53	99.82	99.97	99.99	64.90	88.56	96.34	99.30

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Table 4. Bactericidal efficacy of peracetic acid against test mycobacteria

Test mycobacteria	Average killing rate (%) of PAA concentration (mg/L) after different exposing time (min)											
	200 mg/L				100 mg/L				50 mg/L			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min
M. phlei	99.93	99.99	100	100	98.71	99.82	99.98	99.99	86.07	98.84	99.75	99.97
M. fortuitum	99.94	99.99	100	100	97.72	99.81	99.98	99.99	85.07	98.17	99.74	99.96
M. smegmatis	99.97	100	100	100	99.49	99.97	99.99	100	94.71	99.50	99.91	99.98
M. diernhoferi	99.95	100	100	100	98.50	99.90*	99.99	100	84.11	98.46	99.70	99.96
M. chelonae subsp. abscessus	99.97	100	100	100	98.06	99.75	99.98	99.99	86.46	98.28	99.77	99.98
M. flavescens	99.97	99.99	100	100	99.23	99.95	99.99	100	93.62	99.37	99.91	99.99
M. nonchromogenicum	99.95	99.99	99.99	100	98.46	99.86	99.99	99.99	86.50	98.79	99.82	99.97
M. tuberculosis H37Ra	99.95	99.99	99.99	100	98.52	99.93	99.99	100	86.98	99.33	99.92	99.99
M. xenopi	99.99	99.99	100	100	99.08	99.94	99.99	100	90.45	99.43	99.93	99.99
M. bovis	99.98	99.99	100	100	99.16	99.97	100	100	90.21	99.56	99.94	99.99

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Table 5. Bactericidal efficacy of ethanol against test mycobacteria

Test mycobacteria	Average killing rate (%) of ethanol concentration (%)after different exposing time (min)											
	60				45				30			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min
M. phlei	99.99	100	100	100	99.38	99.93	99.99	100	66.56	83.60	93.85	97.43
M. fortuitum	99.97	100	100	100	99.32	99.93	99.99	100	54.74	79.81	90.48	96.79
M. smegmatis	100	100	100	100	99.24	99.99	100	100	65.07	86.94	96.80	98.75
M. diernhoferi	99.99	100	100	100	99.23	99.96	99.99	100	61.59	82.91	92.60	98.55
M. chelonae subsp. abscessus	99.99	100	100	100	99.75	99.99	99.99	100	59.25	80.85	90.92	97.76
M. flavescens	99.99	100	100	100	99.14	99.94	99.99	100	64.30	84.55	96.01	98.83
M. nonchromogenicum	99.99	100	100	100	99.22	99.94	99.99	99.99	59.44	81.47	93.47	98.43
M. tuberculosis H37Ra	99.99	100	100	100	99.75	99.99	99.99	100	63.16	86.25	96.35	98.47
M. xenopi	99.99	100	100	100	98.77	99.81	99.98	100	60.19	80.58	92.87	98.23
M. bovis	99.94	100	100	100	98.71	99.83	99.98	99.99	56.44	79.14	93.21	97.91

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Table 6. Bactericidal efficacy of TC-101 against test mycobacteria

Test mycobacteria	Average killing rate (%) of available chlorine concentration (mg/L) after different exposeing time (min)															
	80 mg/L				40 mg/L				20 mg/L				10 mg/L			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min
M. phlei	-*	-	-	-	98.27	99.93	99.99	100	90.31	99.22	99.88	99.99	74.83	91.15	99.00	99.82
M. fortuitum	96.54	99.90	99.99	100	88.93	98.71	99.84	99.98	67.33	88.20	98.87	99.72	-	-	-	-
M. smegmatis	-	-	-	-	99.58	99.98	100	100	97.75	99.63	99.95	99.99	76.48	95.63	99.56	99.97
M. diernhoferi	-	-	-	-	98.89	99.94	99.99	100	90.98	98.94	99.84	99.98	69.31	90.67	99.07	99.91
M. chelonae subsp. abscessus	98.00	99.92	99.99	100	89.36	98.98	99.86	99.99	69.85	89.34	98.88	99.83	-	-	-	-
M. flavescens	-	-	-	-	99.13	99.94	99.99	100	97.43	99.52	99.93	99.99	75.15	94.25	99.23	99.94
M. nonchromogenicum	-	-	-	-	98.06	99.92	99.99	99.99	91.16	98.95	99.84	99.99	68.45	90.33	99.13	99.90
M. tuberculosis H37Ra	-	-	-	-	99.75	99.99	100	100	98.03	99.75	99.98	99.99	89.47	98.55	99.77	99.99
M. xenopi	-	-	-	-	99.49	99.96	99.99	100	94.96	99.45	99.94	99.99	74.32	95.96	99.53	99.94
M. bovis	-	-	-	-	99.52	99.98	99.99	100	95.67	99.49	99.95	99.99	71.38	96.94	99.58	99.95

*: means not test.

Apparently, different test conclusion will be given to a given disinfectant by different method, which will puzzle the users. It is of great significance to use a wide-accepted standard evaluation tests in order to get a wide-accepted conclusion.

The most significant thing to standardize the mycobactericidal test is to choose a reference mycobacterial strain. Because of its important status in mycobacterial infection, M. tuberculosis was widely used as the test strain in most mycobactericidal tests. But its high pathogenicity and low growing speed bring great inconvenience to conducting the test. An ideal test mycobacterial strain should be rapid growing, comparatively resistant to disinfectants and safe to the conductors and environment. In this study, we compared the susceptibilities of M. tuberculosis and some rapid growing NTMs by using a suspension quantitative bactericidal test. The results showed that M. chelonei subsp. abscessus and M. fortuitum are as resistant to the tested disinfectants as the other 8 tested mycobacteria or even more resistant, which suggests that M. chelonei subsp. abscessus and M. fortuitum have the potential to be used as reference strain in mycobacteicidal test. This result is consistent with that from Corinne Le Dantec et al [4].

Our study results also showed that some frequently used disinfectants such as iodine, chlorine, glutaraldehyde, ethanol, peracetic acid and cresol disinfectants can effectively kill mycobacteria under concentrations lower than their routinely used concentrations. These results we got in this study were different with those from previous literatures (5-7), which need much higher disinfectants' concentrations. The possible reason, we think, lies in the difference in test methods. In this study, the quantitative bactericidal tests were conducted in a suspension without organic matters and tap water, while in the previous reports organic matters and tap water were used. Since organic matters can react to some disinfectants and provide protection for bacteria, so it can reduce disinfectants' bactericidal efficacy. Tap water can reduce disinfectants' bactericidal efficacy as well. These make the difference in results between our study and previous reports reasonable.

Ethanol was and still is a widely and frequently used disinfectant. In this study, we found that 45% (v/v) of ethanol, much lower than the commonly used concentration, and still possess the mycobactericidal potential. And this concentration is not higher than that have bactericidal effect against S. aureus and E. coli. The cell wall permeability may account for the result. Mycobacterial cell walls have high lipid's content, so lipid soluble disinfectants like ethanol can permeate the cell walls more easily to function on the targets.

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Chapter 22

A Comparison of the Microbicidal Efficacy on Germ Carriers of Several Tertiary Amine Compounds versus OPA and Perasafe

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Several tertiary amine formulations have been marketed as high-level disinfectants (HLD). This study compares some of these formulations with two accepted HLD (OPA and Perasafe), using various methods.

1) determination of the bactericidal effect on 52 microorganisms, using a metallic germ carrier

2) Sporicidal effect using a commercial germ carrier (3M spores)

3) Corrosion test on surgical blades plus human blood.

It should be noted that, despite acting for only 10 minutes, OPA and Perasafe (not differing from each other) are significantly more effective than all other products tested, despite their longer contact (15 and 20 minutes), both for microorganisms overall and by groups. However, as an exception, Instrunet FA showed no significant differences at 20 minutes as compared to OPA and Perasafe at 10 minutes for Gram-negative microorganisms. On the other hand, except for this last data, the tested amines did not significantly differ in their bactericidal efficacy. On the other hand, only OPA and Perasafe were effective against mycobacteria (though the contact time had to be increased to about 15 minutes), but did not reach the sporicidal level. Finally, only one of them (Ultradesmit) altered the metallic material in the corrosion test.

Among all disinfectants tested in this study, only OPA and Perasafe can be considered as HLD, but an action time of 15-20 minutes should be recommended, and it should acknowledged that both products are not free from disadvantages.

Introduction

Tertiary amines are described as surface-active agents with an increased efficacy against *Mycobacteria,* (1) and could therefore be used as high level disinfectants (HLD) provided their efficacy was maintained against all other bacteria and this mycobactericidal action did not only cover *M. tuberculosis* (as killing this bacterium alone would only imply a medium level disinfection), but also other more resistant *Mycobacteria* (some of them atypical) or spores (2-7).

On the other hand, these tertiary amines are associated to other surface-active compounds, usually quaternary ammonia such as benzalkonium chloride, and the mixtures are not always similar, so that efficacy may vary between the different commercial formulas. It must therefore be verified which is most effective and whether all or some of them may be classified as HLDs.

We think that to consider a disinfectant as a HLD, it cannot just be accepted that in some tests, with microorganisms in suspension, the disinfectant achieves a 5log reduction of some *Mycobacteria* and other collection microorganisms (ATCC) for several reasons:

1) ATCC microorganisms and strains aged at the laboratory are always more susceptible than those freshly obtained from patients, particularly when these are microorganisms R or polyR to antibiotics (e.g. we obtained with OPA the following log10 reductions in *P. aeruginosa*: with ATCC >5.5, with freshly isolated microorganisms S to antibiotics, 4.8 log10, and for polyR germs, 3.8 log10). Thus, we must always include in our tests freshly isolated R and polyR microorganisms to evaluate the efficacy spectrum against such microorganisms.
2) In addition, these microorganisms are never in suspension when instruments are to be disinfected, but on the smooth or rough surface of the instrument. Suspension tests alone cannot therefore be accepted, and some germ carrier or surface test must always be included to verify efficacy in this situation (the one that will be found in clinical conditions), because differences between methods with microorganisms in suspension or on germ carriers may be of several log10 (8,9)
3) Finally, since different mycobacteria and viruses cannot be studied in most laboratories, it is appropriate to include a sporicidal test, because if the disinfectant passes this test, efficacy can be extrapolated to other

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not tested mycobacteria and viruses, with or without lipid envelope, which are known to be less resistant than spores. However, if a disinfectant does not pass the sporicidal test it should not be dismissed, because in principle HLDs do not have to be able to kill these resistance forms, but we then must test their efficacy against the more resistant mycobacteria and a substantial number (at least 30) of other bacteria from different species before classifying them as HLDs.

Therefore, we will use in this study microorganisms freshly obtained from patients and other collection microorganisms. Using a surface test, we will compare several tertiary amine formulations with two disinfectants used in our country as HLDs. Such disinfectants include OPA (0.55% ortho-phthalaldehyde, highly effective against mycobacteria and thus recommended as an adequate HLD, though its efficacy against spores is very low), and Perasafe (a powder compound that is diluted in water at the time of use and releases peracetate ions at an approximate 0.25% concentration) (10-12).

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Materials and Methods

Materials

1) Disinfectants:
   - 0.55% Ortho-phthalaldehyde (Johnson & Johnson),
   - 1,65% Perasafe (Lab Antec Int),
   - 4% Korsolex-plus (didecyl-dimethyl-ammonium-chloride 13%, dodecyl-bis-prpylentriamin 9,2%), Lab Bode.
   - Instrunet FA (didecyl-dimethyl-ammonium-chloride 0,7%, diamino-propyl-dodecyl-amin 0,6%, ethanolamine 1,4%): Lab Inibsa, in two versions, one with the already diluted product and certain excipients that increase its efficacy, and another version in which product should be diluted to 5% before use),
   - 12% Sinaldehyd (aminopropyl-dodecylamin 9,9%, didecyl-methyl-polyoxi-ethyl-ammonium-propionate 12%, Lab J Collado),
   - 4% Ultradesmit (dodecyl-bis-propilen-triamin 21%, didecyl- dimethyl-ammonium- chloride 14%, Lab Steer-Pharma).
2) 52 microorganisms: (obtained from ICU patients) from different genera including *Staphylococcus*, *Streptococcus*, *Enterobacteriaceae*, *Pseudomonas*, *Candida*, *M. fortuitum*, and *M. avium* complex (only against OPA and Perasafe). 2 additional collection strains, Mycobacterium smegmatis 155 mc^2 and *B. subtilis* (3M) spores.
3) Endodoncy files no. 25
4) Glass beads (0.25 mm in diameter)
5) Surgical blades

6) Inhibitor of antiseptic action: Todd Hewitt broth (Difco) + 6% v/v Tween 80, 0.5% w/v sodium bisulphite and 0.5% w/v sodium thiosulphate.

All culture media and Tween 80 used in this research were purchased from Johnson & Johnson, Tedec-Meiji Lab and Inibsa Lab through FUAM (Fundacion Universidad Autonoma Madrid).

Methods

1) determination of the bactericidal effect using a metallic germ carrier (no. 25 endodoncy files) (13).

Strains recently isolated from ICU patients or ATCC strains. Endodoncy files were contaminated with a suspension of microorganisms (10^{7-8} CFU/ml) by immersion for one hour before being left to dry (15 minutes) on a sloping sterile surface (Petri dish with no culture medium). Each file was then placed in a tube with 5 ml of the disinfectant for different times: ranging from 1-20 minutes. After this, the files were removed, placed into another tube containing 5 ml of inhibitor with 0.5 g of glass beads 1 mm in diameter, and shaken in a Vortex at 1000 rpm for 1 minute. Finally, two 0.1 ml samples of the supernatant were cultured on Mueller-Hinton plates (or on Sabouraud-dextrose for yeasts or Coletsos medium for Mycobacteria) and incubated at 37°C for 24-48 hours (or 4-15 days for Mycobacteria) to count the number of microorganisms surviving after exposure to the disinfectant. The number obtained was compared with the one obtained for the control (same method but introducing the germ carrier in sterile distilled water instead of disinfectant), and 1/100 and 1/10000 dilutions were made in order to facilitate CFU counts.

The assay was performed for all the microorganisms described in the Materials and Methods section.

2) Sporicidal effect using a commercial germ carrier (3M spores) (14): *B. subtilis* spores were removed from the capsule in which they are supplied and the cellulose impregnated with them was used as a germ carrier that was placed in the disinfectant and left there for 10 or 20 min. Cellulose was subsequently removed and placed in the inhibitor to stop the sporicidal effect. The rest of the assay continued as in the surface test.

3) *Corrosion test*: This was a modification of a previously described test (14). The lancet blade was impregnated with human blood (from a blood bank) to promote deterioration of the blade and mimic a defective cleaning situation. The blade was then introduced in disinfectant and examined for deteriorated areas or global deterioration at 1, 2 , 4, 8, and 24 h, and daily up to 1 week.

4) *Statistical method:* The log10 reduction in the CFU count compared to the control is considered the "effect" of the disinfectant. For disinfections with no surviving organisms, in which the log10 reduction cannot be calculated, a value of 5.5 was recorded. The minimum bactericidal effect for this rough germ carrier test is 3.5 for most bacteria and 3 for *Mycobacteria* (13). For the

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sporicidal test, the threshold established in UNE standards to consider a product as "sporicidal" of 4 log10 is applied.

The effects of the different products were compared using analysis of variance with Bonferroni's or Dunnett's correction depending on whether or not homogeneity of variances was found. The "final effect" at the maximum time considered in the comparison of all amines and the two reference disinfectants, that is, 10 min for OPA and Perasafe and 20 min for all others, was also compared.

Results

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The first comparison was performed in order to determinate the compared speed of kill microorganisms between a classic tertiary amine (Korsolex) and the two most effective reference products used in this study (OPA and Perasafe).

Table 1 shows the bactericidal effect of these three products at between 1 and 15 minutes against 27 microorganisms. Overall, it should be noted that they all have a greater efficacy against Gram-positive organisms, followed by Gram-negative organisms (particularly at 1-5 min), and show a less effective action against *M fortuitum*.

The good efficacy of OPA against almost all microorganisms tested should be noted, as it rapidly increases from 1 min, and total killing of the inoculum is achieved at 10 min. For *M. fortuitum*, however, a slower increase in efficacy is seen, and 15 min are required to achieve a substantial mycobactericidal effect (approximately 4 log10).

Perasafe acts similarly, with no significant differences from OPA between the trends for the different microorganisms, and even the lower efficacy of both disinfectants against *M. fortuitum* almost overlaps. The only difference that can be stressed is maybe the greater efficacy of Perasafe at short times (1-5 min) against Gram-positive organisms, but no significant differences are seen in the tested time points overall.

However, 4% Korsolex consistently has a lower bactericidal effect as compared to the other two products, and at the longest time tested does not achieve total killing of the microbial inoculum in any case, though it achieves the minimum efficacy of 3.5 log10 against *Staphylococcus* and NFB other than *P. aeruginosa*. Korsolex does not also reach the 3 log10 threshold against *M. fortuitum*, and the bactericidal trend also expresses a "depletion" of efficacy against mycobacteria, since little increase is seen in log10 reduction even when exposure time is greatly increased.

Table 2 simultaneously compares all 7 products in a determinate point of time: 10 minutes, Perasafe and OPA and tertiary amines were evaluated at two different times, since some manufacturers recommend 15 minutes and others, 20 minutes.

The following results must be noted:

OPA and Perasafe completely kill the microbial inocula of Gram-positive

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Table 1: Speed of the bactericidal effect between 1 and 15 minutes of 3 disinfectants against various microorganisms: mean log10 reduction at each time point.

Microorganisms and Products	*time points*			
	1′	*5′*	*10′*	*15′*
MRSA (2) and *MSSA* (2)				
OPA	2	4	5.5	5.5
Perasafe	4	5.5	5.5	5.5
4% Korsolex	1.9	3.4	3.8	3.9
S. coag- (2) and *S. faecalis* (2)				
OPA	2.6	5	5.5	5.5
Perasafe	5	5.5	5.5	5.5
4% Korsolex	2.9	3.5	3.9	5
Klebsiella (2), *Enterobacter* (2), and *Serratia* (2)				
OPA	3.1	4.8	5.5	5.5
Perasafe	2.6	4.6	4.9	5.1
4% Korsolex	1.1	2.1	4.5	3.9
E. coli (2) and *P. mirabilis* (2)				
OPA	2.6	4.4	5.5	5.5
Perasafe	3.34	3.8	4.4	4.7
4% Korsolex	0.8	2	2.5	3.2

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P. aeruginosa S (2) and *P. aeruginosa* polyR (2)				
OPA	3.1	4.3	5.5	5.5
Perasafe	2.6	3	4.2	4.8
4% Korsolex	2.2	2.6	2.9	3.1
Other NFBs (4)				
OPA	3.3	4.5	5.5	5.5
Perasafe	3	4	4.8	5.1
4% Korsolex	2.5	2.9	3.8	4.3
M. fortuitum (1)				
OPA	1.5	2.1	2.95	3.9
Perasafe	1	1.7	3	3.8
4% Korsolex	0.9	1.4	2.1	2.3

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Table 2: Bactericidal effect between 10 and 20 minutes of 7 disinfectants against various Gram positive microorganisms: mean log10 reduction at each time point:

Microorganisms and Products	*time points* *10*	*15'*	*20'*
S. epidermidis (3)			
(Non-dil)Instrunet FA	-	2.8	3.9
5%Instrunet FA	-	2.9	3.7
4%Korsolex	-	4.2	4.8
12%Sinaldehyd	-	3.3	4
4%Ultradesmit	-	3.9	4
Perasafe	5.2 *		
OPA	5.2 *		
S. aureus (3)			
(non-dil)Instrunet FA	-	2.2	2.5
5% Instrunet FA	-	1.5	3
4%Korsolex	-	2.4	2.5
12%Sinaldehyd	-	2.5	3.4
4%Ultradesmit	-	3.7	3.9
Perasafe	4.5 *		
OPA	4.5 *		
S. faecalis (3)			
(non-dil)Instrunet FA	-	4.3 *	4.3 *
5% Instrunet FA	-	2.6	3.5
4%Korsolex	-	3.2	3.5
12%Sinaldehyd	-	3.1	3.6
4%Ultradesmit	-	2.9	3.1
Perasafe	4.3 *		
OPA	4.3 *		
K. pneumoniae (3)			
(non-dil)Instrunet FA	-	5.1	5.1
5% Instrunet FA	-	2.7	3.1
4%Korsolex	-	3.3	3.7
12%Sinaldehyd	-	3	3.2
4%Ultradesmit	-	2.6	2.9
Perasafe	5.2 *		
OPA	5.2 *		

*= inoculum is completely killed Table 2 cont.: Bactericidal effect between 10 and 20 minutes of 7 disinfectants against various microorganisms: mean log10 reduction at each time point:

Table 2. *Continued.* Bactericidal effect between 10 and 20 minutes of 7 disinfectants against various Gram negative microorganisms: mean log10 reduction at each time point:

Microorganisms and Products		*time points*	
	10	*15′*	*20′*
P. mirabilis (3)			
(non-dil)Instrunet FA	-	3.4	5.1 *
5%Instrunet FA	-	1.2	3.3
4%Korsolex	-	2.7	3.6
12%Sinaldehyd	-	2.9	3.3
4%Ultradesmit	-	1	1.2
Perasafe	5.1 *		
OPA	5.1 *		
E. cloacae (3)			
(non-dil)Instrunet FA	-	4.4	5
5% Instrunet FA	-	3.1	3.5
4%Korsolex	-	2.8	3.3
12%Sinaldehyd	-	3.4	3.7
4%Ultradesmit	-	2	2.6
Perasafe	5.2 *		
OPA	5.2 *		
P. aeruginosa (3)			
(non-dil)Instrunet FA	-	4.9	5.2 *
5%Instrunet FA	-	3.7	3.8
4%Korsolex	-	3.8	4
12%Sinaldehyd	-	4.7	4.8
4%Ultradesmit	-	3	3.6
Perasafe	5.2 *		
OPA	5.2 *		
C. albicans (3)			
(non-dil)Instrunet FA	-	2.7	3.2
5%Instrunet FA	-	1.2	1.5
4%Korsolex	-	1.4	3.1
12%Sinaldehyd	-	3.9	4
4%Ultradesmit	-	1.8	2.6
Perasafe	4 *		
OPA	4 *		

*= inoculum is completely killed Table 2 cont.: Bactericidal effect between 10 and 20 minutes of 7 disinfectants against various microorganisms: mean log10 reduction at each time point:

Continued on next page.

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Table 2. *Continued.* Bactericidal effect between 10 and 20 minutes of 7 disinfectants against various Mycobacteria and one spore: mean log10 reduction at each time point:

Microorganisms and Products	*time points* *10*	*15′*	*20′*
M. fortuitum			
Non-dil)Instrunet FA	-	1.6	1.8
5%Instrunet FA	-	1	1.4
4%Korsolex	-	1.7	2.2
12%Sinaldehyd	-	1.2	1.5
4% Ultradesmit	-	0.6	1.2
Perasafe	3.3	3.5*	
OPA	3.1	3.5*	
M. smegmatis mc^2 155			
(non-dil)Instrunet FA	-	2.1	3.9
5% Instrunet FA	-	1.7	2.7
4%Korsolex	-	2.7	2.8
12%Sinaldehyd	-	3	3.5
4%Ultradesmit	-	2.5	3.2
Perasafe	4	4.7*	
OPA	4.7*	4.7*	
M. avium-complex			
(non-dil)Instrunet FA	-	-	-
5%Instrunet FA	-	-	-
4%Korsolex	-	-	-
12%Sinaldehyd	-	-	-
4%Ultradesmit	-	-	-
Perasafe	4.9	4.9*	
OPA	4.9*	4.9*	
B. subtilis spores (ATCC)			
(Non-dil)Instrunet FA	-	0.7	1.3
5% Instrunet FA	-	0.2	1.3
4% Korsolex	-	0.3	0.9
12% Sinaldehyd	-	0.3	1.2
4% Ultradesmit	-	0.3	0.6
Perasafe	2.9		
OPA	1.2		

*= inoculum is completely killed Table 2 cont.: Bactericidal effect between 10 and 20 minutes of 7 disinfectants against various microorganisms: mean log10 reduction at each time point:

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cocci, Gram-negative bacilli, and yeasts, while for *M. smegmatis* and *M. avium* complex they reach more than 4 log10, but for *M. fortuitum* they barely exceed the minimum threshold of 3 log10. On the other hand, they show a lower efficacy against spores, with reductions of just over 1 log10 for OPA and almost 3 log10 for Perasafe.

Overall, these are the most effective products tested, but there are no significant differences between them.

As regards tertiary amines, we should note that the formulation of Instrunet FA requiring no dilution was more effective than the one that should be diluted before use. A comparison of this undiluted formula to all other amines shows the following:

The most effective products against *S. epidermidis* are Korsolex and Ultradesmit, that reach the 3.5 log10 threshold in 15 minutes, while against S. aureus only the latter formula reaches this critical point from 15 minutes. The opposite occurs with *S. faecalis*, since Ultradesmit does not exceed a 3.5 log10 reduction in 20 minutes, unlike all other tertiary amines.

Against Gram-negative microorganisms, Instrunet FA is most effective, achieving over 5 log10 in 20 minutes, while all other product barely reach the required threshold, except against *P. aeruginosa*, which is the Gram-negative microorganism most susceptible to these tested product. Against yeasts, only Sinaldehyd achieves 3.5 log10, both a 15 and 20 minutes.

All preparation, except Korsolex and Instrunet FA, are effective in 20 minutes against M. smegmatis, but none of them achieves 3 log10 against *M. fortuitum*. Due to such low efficacy for the latter microorganism, the effect of these amines against *M. avium* complex was not tested.

Finally, all amines are poorly sporicidal in 20 minutes, as they only kill 0.6-1.3 log10.

Table 3 compares statistically the bactericidal effects of the used products against microorganisms, overall and grouped as Gram-positive microorganisms (*S. epidermidis, S. aureus, E. faecalis*, and *C. albicans*), Gram-negative bacteria (*P. aeruginosa, Proteus, Enterobacter*, and *Klebsiella*), *Mycobacteria* (*M. fortuitum* and *M. smegmatis* or, for OPA and Perasafe, also including *M. avium* complex), and *B. subtilis* spores.

***subtilis* spores**

It should be noted that, despite acting for only 10 minutes, OPA and Perasafe (not differing from each other) are significantly more effective than all other products tested, despite their longer contact (15 and 20 minutes), both for microorganisms overall and by groups. However, as an exception, Instrunet FA showed no significant differences at 20 minutes as compared to OPA and Perasafe at 10 minutes for Gram-negative microorganisms. On the other hand, except for this last data, the tested amines did not significantly differ in their bactericidal efficacy.

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Table 3. Statistical comparison of the bactericidal effect of different amines versus OPA and Perasafe (log10 reduction in 10, 15, or 20 minutes, depending on the product. Mean and SD).

Products	*All micro organisms*		*Gram+*		*Gram-*		*Mycob*	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
OPA (10 min)	4.63	0.88	4.5	0.46	5.17	0.04	4.3	ND
Perasafe (10 min)	4.67	0.65	4.5	0.46	5.17	0.04	4	ND
Instrunet FA								
undiluted (15 min)	3.47	1.23	3	0.81	4.45	0.69	1.85	ND
(20 min)	4.07	1.16	3.47	0.71	5.1	0.07	2.85	ND
5% Korsolex (15 min)	2.81	0.95	2.8	1.07	3.15	0.45	2.2	ND
(20 min)	3.38	0.83	3.47	0.88	3.65	0.26	2.5	ND
12% Sinaldehyd (15 min)	3.04	1.05	3.2	0.52	3.5	0.74	1.9	ND
(20 min)	3.47	0.97	3.75	0.27	3.75	0.66	2.37	ND
4% Ultradesmit (15 min)	2.46	1.06	3.07	0.86	2.15	0.79	1.8	ND
(20 min)	2.85	0.99	3.4	0.6	2.57	0.91	2.35	ND

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ND = non determined (only n=3)

According to the analysis of variance:
For the microorganisms overall, the efficacy order is:

1. OPA and Perasafe: No significant differences between them
2. Instrunet FA undiluted
3. Sinaldehyd
4. All others: No significant differences between them

For Gram-positive microorganisms, the efficacy order is:

1. OPA and Perasafe: No significant differences between them
2. All amines: No significant differences between them

For Gram-negative microorganisms, the efficacy order is:

1. OPA and Perasafe: No significant differences between them
2. Instrunet FA undiluted: Not different from OPA and Perasafe in 20.
3. Korsolex and Sinaldehyd: No significant differences between them
4. Ultradesmit

For Mycobacteria, the efficacy order is:

1. OPA and Persafe: No significant differences between them
2. All other products testes: No significant differences between them

Thus, we can group the 7 products tested in 3 categories. OPA and Perasafe would be the most effective, followed by Instrunet FA (undiluted) as an amine somewhat more effective than the others, and then all other products tested.

Finally, Figure 1 shows the "most favorable" mean effect of those obtained, i.e. 10 min for OPA and Perasafe or 20 min for all other disinfectants. Breakpoints are plotted, and a visual assessment can be made of what the statistical analysis suggests: among amines, only the undiluted formula of Instrunet FA shows significant differences from the others (due to its greater efficacy against Gram-negative germs), but they are overall similar. However, they all are less effective that OPA and Perasafe. Moreover, this greater efficacy is achieved by OPA and Perasafe after only 10 min, as compared to 20 min with amines.

They did not alter the lancet blades with human blood in 7 days (equivalent to more 500 continuous disinfections of 20 min each in the case of amines and to more 1000 for OPA and Perasafe), except for Ultradesmit, which oxidizes the blade in one point (about 1 mm^2) after the first day.

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Discussion

When the different microorganisms S or R to antibiotics (freshly isolated from patients) are tested on rough germ carriers, it may occur that a disinfectant considered as a HLD does not exceed the threshold required by this surface test against the specific microorganism, although such microorganism should theoretically be killed by that disinfectant to maintain its HLD label. For this we must verify the efficacy in a high number of microorganisms, but there is always a possibility that such decreased efficacy occurs in a certain number of them. We think that this problem should be addressed by proposing that the number of microorganisms for which efficacy is decreased does not exceed 0.5% when they are randomly selected from non-ICU patients, or 1% if they come from ICU patients (microorganisms probably selected by antibiotic therapy). Such cases would result in disinfection failure, but it should be taken into account that this would require another concomitant failure in washing before disinfection. However, this does not mean that an infection will necessarily occur, because microorganisms must previously overcome antagonism by the endogenous flora and immune system of the patient in whom instruments with such failed disinfection are introduced. Nonetheless, we must avoid that risk as much as possible.

Efficacy of the different tertiary amines is quite similar when globally considered, and the optimum time of use is 20 min, as there are significant differences between 15 and 20 min. However, if one of them should be chosen, the most effective appears to be the Instrunet FA solution requiring no dilution, probably because of its excipients, that greatly increase its pH and place the formulation in a better position to achieve the best performance among all ammonia compounds used.

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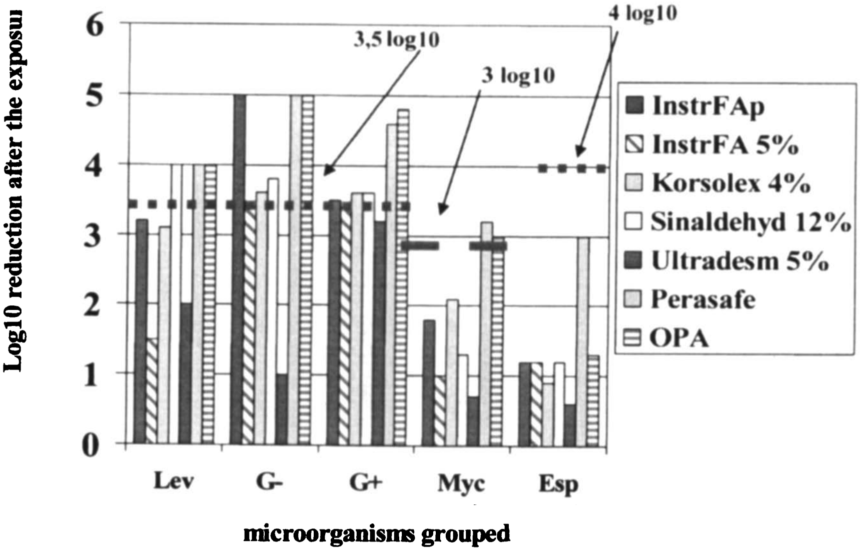

Figure 1. Global comparison between disinfectants (OPA and Perasafe, 10 min versus various tertiary amines, 20 min)

Efficacy may however be considered from a qualitative viewpoint, that is, evaluating whether the level required to consider them as HLDs is reached. Such level implies the ability to kill microorganisms more susceptible than spores, including all *Mycobacteria*. This is very difficult to assess, since it depends on the method selected, and even the disinfectant most widely accepted as HLD, 2% glutaraldehyde, is not affective in 20 min against *M avium-intracellulare*, but requires an increase in exposure time to 45 min. Nevertheless, certain *Mycobacteria*, including at least one that is more resistant than *M. tuberculosis* such as *M. fortuitum*, should at least be considered in a germ carrier test, because microorganisms to be disinfected in HLDs will actually be on various germ carriers with a smooth of rough surface. In our case, there is not much difference in using any of both germ carriers if we establish different efficacy thresholds, that is, if the European germ carrier test (using a smooth glass plate) requires a 4 log10 reduction and our test (using rough metal and plastic) only requires a 3 log10 reduction (13)

From this viewpoint, tertiary amines used in this study should not be classified as HLDs both based on tests with other microorganisms more susceptible than *Mycobacteria* and on their action against *Mycobacteria*, and only OPA and Perasafe meet the requirements to be considered HLDs: 1) more than 3 log10 *Mycobacteria* killed, and 2) among ICU microorganisms, less than 1% show more survivors than the critical threshold, 3.5 log10 (0% for both these products).

These products could participate in a HLD "process" if an additional stage was added to it, consisting of flooding with 100 ml of product before leaving the endoscope channels filled and starting the disinfection process proper (9) Thus, most disinfection controls conducted at our hospital have been negative (despite use of a disinfectant not having a high efficacy against *Mycobacteria* and which could not be adequately considered as a high level disinfectant), provided all other steps have been adequate, but in a minimum percentage (less than 2% in our samples) this does not occur for several reasons, and disinfection may then fail. Hence, disinfectants achieving by themselves an appropriate reduction of microorganisms, regardless of the rest of the disinfection process, should be used. We therefore think that among all disinfectants analyzed, only OPA and Perasafe should be included in high level disinfection protocols at our hospitals, because they do not only kill all tested microorganisms, but can also kill *M avium-intracellulare* (11,12) though it should be reminded that OPA cannot be used in urology and stains the areas where it is poured (clothes, etc.), and Perasafe is difficult to dilute if the water used is not at an adequate temperature (about 40°C), and both this and its permanence in the disinfection tray allows for peracetic evaporation, and the resulting smell has been sometimes poorly accepted by the exposed health care personnel. An additional problem reported for this product is an impaired articulation of instruments such as biopsy forceps, probably due to oxidation of this point of instruments.

We also think that after 10 minutes both products are too near the "non efficacy" level with the *M. fortuitum* used, and could have failed the test if a

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more resistant microorganism had been used. However, in the study where exposure time was increased to 15 minutes (Table 1), an increased efficacy was shown as compared to 10 minutes, allowing for a substantial safety margin with both Perasafe and OPA. This agrees with some guidelines (15-20) that recommend a time longer than 10 minutes for HLD with OPA, and 15 min was also the time recommended by us for Perasafe in a prior publication (14)

Efficacy on spores has been tested in order to extrapolate conclusions to other untested microorganisms, that is, if a sporicidal effect had been obtained (>4 log10 reduction) (21), we could infer that the products would also be effective against other untested *Mycobacteria* particularly resistant to certain disinfectants (such as glutaraldehyde, the reference HLD product, which is however more effective against mycobacteria, killing them in 25 min, while it takes 3-10 h to have a sporicidal effect) o against viruses (*Enterovirus, Coronavirus, Myxovirus*, etc, that may be present in endoscopes) (22,23). However, such extrapolation is not possible because of the low sporicidal power of most tested products, although Perasafe may approach this induction, particularly when used with an exposure time of 15 instead of 10 minutes.

Finally, there are other factors related to the two products we consider HLDs to be taken into account, namely: 1) changes in the material on which they act (they both appear to be similar in this regard, except for the abovementioned cases of biopsy forceps), and 2) price, which is in principle higher for OPA, but may actually be the opposite, taking into account Perasafe should be discarded daily, while OPA may act for up to 14 days, and also has an adequate indicator that would allow for product disposal before the expected data if it has degraded more than usual with use. This is a control mechanism that should be required for any disinfecting solution that is claimed to last for longer than one day.

To conclude, among all disinfectants tested in this study we only can include OPA and Perasafe as high level disinfectants, but recommending an action time of 15-20 minutes and taking into account that they are not free from disadvantages (staining and avoidance of use in urology for OPA, and a strong smell of vinegar, occasional oxidation of instruments, and need to discard solution every day, which may ultimately make the process much more expensive for Perasafe). Thus, research must be continued in this field to obtain products that come closer to the characteristics of the ideal HLD.

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Prion = Proteinaceous Infectious Particle

PrP^c = Prion Protein cellular =normal or "healthy" prions

PrP^{sc} = rogue prions/ pathologic isoform

PrP^c and PrP^{sc} are the same chemical but have different shapes

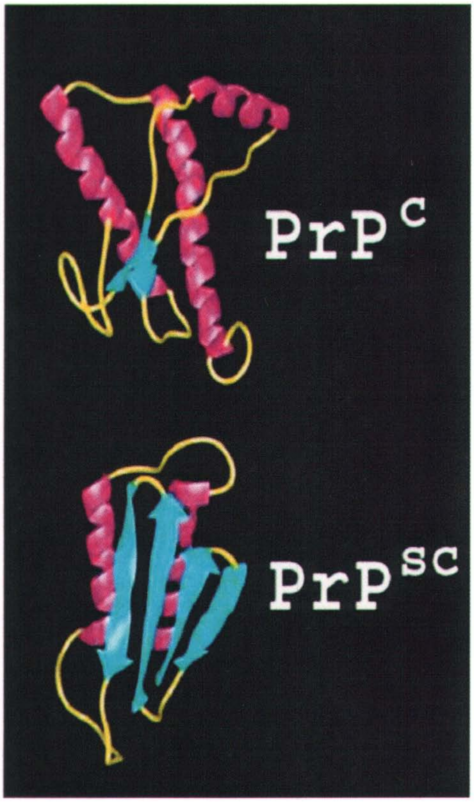

Figure 4.1. Images of Prions.

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